Chloride is an Agonist of Group II and III Metabotropic Glutamate Receptors

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ABSTRACT

The elemental anion chloride is generally considered a passive participant in neuronal excitability, and has never been shown to function as an agonist in its own right. We show that the antagonist-mediated, glutamate-independent inverse agonism of group II and III metabotropic glutamate (mGlu) receptors results from inhibition of chloride-mediated activation. In silico molecular modeling, site-directed mutagenesis, and functional assays demonstrate (1) that chloride is an agonist of mGlu3, mGlu4, mGlu6, and mGlu8 receptors with its own orthosteric site, and (2) that chloride is not an agonist of mGlu2 receptors. Molecular modeling–predicted and site-directed mutagenesis supported that this unique property of mGlu2 receptors results from a single divergent amino acid, highlighting a molecular switch for chloride insensitivity that is transduced through an arginine flip. Ultimately, these results suggest that activation of group II and III mGlu receptors is mediated not only by glutamate, but also by physiologically relevant concentrations of chloride.

Introduction

Cells in the central nervous system are functionally dependent on fluctuations of elemental ions. While endogenous cations participate in propagating action potentials, they can also directly modulate receptor activity. For example, calcium flux through N-methyl-d-aspartate receptors influences neuronal depolarization (Traynelis et al., 2010), modulates metabotropic glutamate (mGlu) 1 receptor activation (Jiang et al., 2014), and activates the Ca\(^{2+}\)-sensing receptor (Brown et al., 1993). In contrast, the elemental anion chloride is generally considered an essential but passive participant in neuronal excitability, and to our knowledge it has never been shown to function as an agonist of any receptor in its own right. However, chloride has been shown to modulate receptor function. For example, the kainate receptors require a chloride ion between protomers to facilitate dimerization (Chaudhry et al., 2009). It has also been shown that the binding of atrial natriuretic peptide (ANP) to ANP receptors requires chloride (Misono et al., 2011), and that the presence of chloride influences ANP receptor oligimerization (Ogawa et al., 2010). Interestingly, it has been reported that the ANP receptor chloride binding site is structurally conserved at mGlu receptors (Ogawa et al., 2010; Acher et al., 2011).

mGlu receptors are a family of class C G protein–coupled receptors, which is subdivided into three groups based on pharmacology, sequence homology, and signal transduction. While group I [mGlu1 and mGlu5 (IUPHAR = mGlu1 and mGlu5)] receptors are G\(_4\)-coupled, group II [mGlu2 and mGlu3 (IUPHAR = mGlu2 and mGlu3)] receptors and group III [mGlu4, mGlu6, mGlu7, and mGlu8 (IUPHAR = mGlu4, mGlu6, mGlu7, and mGlu8)] receptors are G\(_i/o\)-coupled (Conn and Pin, 1997; Alexander et al., 2013). While the literature is consistent regarding calcium-mediated modulation of mGlu1 receptors (Saunders et al., 1998; Jiang et al., 2014), the contribution of ions to group II and III mGlu receptor signaling is less clear. For example, one study concluded that mGlu3 receptors are activated by calcium (Kubo et al., 1998), while another study claims that ZnCl\(_2\), not calcium, affects our knowledge it has never been shown to function as an agonist of any receptor in its own right. However, chloride has been shown to modulate receptor function. For example, the kainate receptors require a chloride ion between protomers to facilitate dimerization (Chaudhry et al., 2009). It has also been shown that the binding of atrial natriuretic peptide (ANP) to ANP receptors requires chloride (Misono et al., 2011), and that the presence of chloride influences ANP receptor oligimerization (Ogawa et al., 2010). Interestingly, it has been reported that the ANP receptor chloride binding site is structurally conserved at mGlu receptors (Ogawa et al., 2010; Acher et al., 2011).

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radioligand binding at mGlu2, but not mGlu3, receptors (Schweitzer et al., 2000). In previous work, we reported that the removal of chloride increased agonist efficacy at mGlu3 receptors (DiRaddo et al., 2014). As a confounding variable, group II and III mGlu receptors have been notoriously difficult to study (Schweitzer et al., 2000; Niswender et al., 2008; DiRaddo et al., 2014), resulting in inconsistent reports of ligand potency and affinity (Conn and Pin, 1997; Schoepf et al., 1999), and several accounts of inverse agonism (Ma et al., 1997; Suzuki et al., 2007; DiRaddo et al., 2014).

Because mGlu receptors are important regulators of neuronal signaling and promising drug targets, we examined the effects of chloride on group II and III receptors. We found that the antagonist-mediated inverse agonism of group II and III mGlu receptors, observed in transfected Chinese hamster ovary (CHO) cells and primary cultures of rat cerebellar astrocytes, is actually due to functional competition with chloride. Using a combination of a recently reported, real-time cAMP assay (DiRaddo et al., 2014), site-directed mutagenesis, and in silico molecular modeling, we discovered that chloride is an agonist at mGlu3, mGlu4, mGlu6 and mGlu8 receptors, while mGlu2 receptors uniquely are not activated by chloride. Furthermore, we confirm the presence of an orthosteric chloride site, and describe the structural basis of mGlu2 receptor chloride insensitivity.

Materials and Methods

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**Forskolin, l-glutamic acid, 2-(1S,2S)-2-carboxyclopropyl)-3-(9H-xanthen-9-yl)-1-alanine (LY341495), (2S)-α-ethylglutamic acid, (RS)-α-cyclopropyl-4-phosphonophenylglycine, and pertussis toxin (PTX) were purchased from Tocris Bioscience (Ellisville, MO). Pyruvate, NaCl, KCl, CaCl2, MgCl2, NaHCO3, HEPES, NaGlucinate, KGlucinate, CaGlucinate2, MgGlucinate2, NaMeSO3, KMeSO3, gluconic acid, proline, and Techno Plastic Products (TPP) tissue culture plasticware were purchased from Sigma-Aldrich (St. Louis, MO). >-Luciferin potassium salt was purchased from Gold Biotechnology (St. Louis, MO). Dubecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), antibiotic/antimycotic, and dialyzed FBS for tissue cultures were purchased from Invitrogen (Carlsbad, CA). Glutamate pyruvate transaminase (GPT) was purchased from Roche Diagnostics (Indianapolis, IN). All buffers were made using ultrapure H2O from a Milli-Q water purification system (EMD Millipore, Billerica, MA).

**Cell Cultures.** CHO cells were transfected with pGloSensor-22F CHO cells were transfected with pGloSensor-22F plasmid (Promega, Madison, WI), and a stable cell line was obtained (CHO-Glo). CHO-Glo cells were then transfected with pIRES-AcGFP1 CHO-Glo cells were then transfected with pIRES-AcGFP1 Cell Cultures. CHO cells were transfected with pGloSensor-22F plasmid (Promega, Madison, WI), and a stable cell line was obtained (CHO-Glo). CHO-Glo cells were then transfected with pIRES-AcGFP1 Cell Cultures. CHO cells were transfected with pGloSensor-22F plasmid (Promega, Madison, WI), and a stable cell line was obtained (CHO-Glo). CHO-Glo cells were then transfected with pIRES-AcGFP1 Cell Cultures. CHO cells were transfected with pGloSensor-22F plasmid (Promega, Madison, WI), and a stable cell line was obtained (CHO-Glo). CHO-Glo cells were then transfected with pIRES-AcGFP1 Cell Cultures. CHO cells were transfected with pGloSensor-22F plasmid (Promega, Madison, WI), and a stable cell line was obtained (CHO-Glo). CHO-Glo cells were then transfected with pIRES-AcGFP1 Cell Cultures. CHO cells were transfected with pGloSensor-22F plasmid (Promega, Madison, WI), and a stable cell line was obtained (CHO-Glo). CHO-Glo cells were then transfected with pIRES-AcGFP1 Cell Cultures. CHO cells were transfected with pGloSensor-22F plasmid (Promega, Madison, WI), and a stable cell line was obtained (CHO-Glo). CHO-Glo cells were then transfected with pIRES-AcGFP1 Cell Cultures. CHO cells were transfected with pGloSensor-22F plasmid (Promega, Madison, WI), and a stable cell line was obtained (CHO-Glo). CHO-Glo cells were then transfected with pIRES-AcGFP1 Cell Cultures. CHO cells were transfected with pGloSensor-22F plasmid (Promega, Madison, WI), and a stable cell line was obtained (CHO-Glo). CHO-Glo cells were then transfected with pIRES-AcGFP1 Cell Cultures. CHO cells were transfected with pGloSensor-22F plasmid (Promega, Madison, WI), and a stable cell line was obtained (CHO-Glo). CHO-Glo cells were then transfected with pIRES-AcGFP1 Cell Cultures. CHO cells were transfected with pGloSensor-22F plasmid (Promega, Madison, WI), and a stable cell line was obtained (CHO-Glo). CHO-Glo cells were then transfected with pIRES-AcGFP1
1993). Using the Prime application within Maestro, the resulting mGlu3 receptor model was used as a three-dimensional template for generating the corresponding mGlu2 receptor homology model. A web-based version of Protein BLAST (National Center for Biotechnology Information, Bethesda, MD) was used for multiple sequence alignment of all eight mGlu receptors, resulting in an mGlu3 and mGlu2 receptor sequence alignment that was 66% identical and 80% homologous. This alignment was imported into Prime, where all sequence gaps were left untreated, except for a three residue gap in the mGlu3 receptor sequence. Secondary structure prediction indicated α-helical character spanning this gap, and accordingly the single displaced α-helical residue (S503 at mGlu3 receptors, see Supplemental Fig. 4) was moved to the other side of the gap. Using Prime, the resulting mGlu2 receptor structure was subjected to a series of loop refinements, energy minimizations, and side chain predictions. Model specifications were again analyzed with web-based ProCheck, and an optimized mGlu2 receptor homology model was generated. The mGlu3 receptor model and corresponding mGlu2 receptor homology model were exported from Maestro as .pdb files, which were subsequently imported into PyMOL for figure preparation (Schrödinger, 2002).

Data Analysis and Statistics. Statistical significance was assessed using a two-tailed Student’s t test or one-way analysis of variance with a Bonferroni post hoc test, as appropriate. Concentration-response curves were calculated by nonlinear regression using the 4-parameter logistic equation. Calculations were performed using GraphPad Prism software (La Jolla, CA).

Results

Inverse Agonism at Group II and III mGlu Receptors Is Not Due to Competition with Glutamate. While establishing the GloSensor cAMP assay to measure activation of the G_{i/o}-coupled mGlu receptors (DiRaddo et al., 2014), we observed that relative to vehicle treatment the nonselective, competitive antagonist LY341495 significantly increased forskolin-stimulated cAMP production in mGlu3-Glo, mGlu4-Glo, mGlu6-Glo, and mGlu8-Glo cell lines, but not in mGlu2-Glo cell lines (Fig. 1, A–E, bars). Although we have successfully expressed mGlu7 receptors, a functional mGlu7-Glo cell line remains elusive (DiRaddo et al., 2014). Pretreatment with PTX completely eliminated the effect of LY341495 (Fig. 1, A–E, bars), indicating that this antagonist-mediated increase in cAMP was due to G_{i/o} signaling, and resulted from a disinhibition of adenylyl cyclase (AC). The concentration-response curves of LY341495, (2S)-α-ethylglutamic acid (a group II-selective antagonist), and (RS)-α-cyclopropyl-4-phosphonophenylglycine (a group III-selective antagonist) demonstrated dose dependency, confirming that inverse agonism is mGlu receptor mediated and not unique to LY341495 (Fig. 1, A–E, curves). These results encouraged us to determine whether LY341495 could disinhibit cAMP production in a native system. Indeed, in primary cultures of cerebellar astrocytes, LY341495 increased forskolin-stimulated cAMP levels, and furthermore glutamate did not significantly inhibit cAMP production (Fig. 1F). This result is consistent with our work in transfected cells (DiRaddo et al., 2014), and could indicate that antagonists of mGlu3, mGlu4, mGlu6, and mGlu8 receptors are actually inverse agonists of high constitutive G protein–coupled receptor activity (~80% at mGlu3 and mGlu8 receptors).

Alternatively, antagonists could have been competing with residual glutamate. Therefore, experiments were performed using GPT, an enzyme that converts glutamate to an inactive metabolite, α-ketoglutarate. The concentration-response curves of LY341495 at mGlu3 receptors showed statistically indistinguishable potencies in the absence (EC50 = 21 nM) and presence (EC50 = 14 nM) of GPT (Fig. 2A). However, in the presence of 30 μM glutamate the concentration-response
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Chloride Activates mGlu3, mGlu4, mGlu6, and mGlu8 Receptors at Its Own Orthosteric Site, Which Is Disrupted at mGlu2 Receptors. We previously showed that chloride replacement (with gluconate) improved agonist efficacy at mGlu3 receptors (DiRaddo et al., 2014). To determine that this effect was, in fact, due to chloride removal and not gluconate addition, experiments were performed using structurally distinct anion substitutions. Substitution of chloride with gluconate or methanesulfonate at all receptors, as well as bicarbonate at mGlu3 receptors, revealed statistically indistinguishable results (Supplemental Fig. 1, A–E), confirming that this effect was chloride specific. To investigate the effects of chloride on the activation of group II and III mGlu receptors, buffers containing increasing concentrations of chloride (replaced with gluconate) were employed. To compare the chloride concentration-response curves between different cell lines, the results were normalized to maximal cAMP production at each chloride concentration (i.e., Fig. 3A versus Supplemental Fig. 2A). Because halides are known to affect AC activity (Kalish et al., 1974), this method of normalization accounts for receptor-independent effects of chloride on AC (see the raw data in Supplemental Fig. 2) and results in concentration-response curves that represent only chloride-mediated activation of the indicated mGlu receptors. As a negative control, a saturating concentration (10 μM) of the orthosteric mGlu receptor antagonist LY341495 was employed to measure receptor-independent cAMP production. Additionally, a saturating concentration of glutamate (1 mM) was used as a positive control to establish the maximal level of mGlu receptor–mediated inhibition of cAMP production. Using this experimental paradigm, increasing chloride concentrations revealed concentration-dependent chloride-mediated activation of mGlu3 (EC50 = 29 mM), mGlu4 (EC50 = ∼280 mM), mGlu6 (EC50 = ∼120 mM), and mGlu8 (EC50 = 21 mM) receptors, but not mGlu2 receptors (Fig. 3, B–F). Standard errors and hill slopes are reported in Supplemental Table 2.

Fig. 2. LY341495-mediated increases in cAMP levels are not due to competition with glutamate. (A) The concentration-response curves of LY341495 in the absence or presence of GPT show no statistical difference in antagonist potency. (B) When spiked with 30 μM glutamate, the concentration-response curves of LY341495 in the absence of GPT show a 9-fold rightward shift in antagonist potency, relative to the presence of GPT. EC50 values of LY341495 were statistically different in the absence versus the presence of GPT. For experiments represented in both panels, relative increases in LY341495 efficacy were observed in the presence of GPT. Cells were incubated in DMEM buffer in the absence or presence of GPT for 4 hours prior to assay. EC50 values were calculated using the 4-parameter logistic equation. Statistical significance was assessed using a two-tailed Student’s t test. Data are presented as the mean ± S.E.M. of three individual experiments performed in triplicate.

Additional experiments revealed that chloride and glutamate inhibit cAMP production to the same degree and that high chloride concentrations produce maximal receptor activation that is neither additive with nor affected by glutamate. We conclude that chloride independently activates mGlu3, mGlu4, mGlu6, and mGlu8 receptors, but not mGlu2 receptors.

We then hypothesized that the previously reported putative chloride binding site (Acher et al., 2011) is, in fact, the orthosteric pocket for chloride. Although no crystal structures of the ECDs of mGlu2, mGlu4, mGlu6, or mGlu8 receptors are available, several agonist-bound mGlu3 receptor ECD crystal structures have been published (Muto et al., 2007). Unfortunately, the corresponding X-ray structures do not show chloride occupation of the putative site; rather, this pocket is occupied by a water molecule. In contrast, an unpublished crystal structure of the mGlu3 receptor ECD bound to LY341495 (PDB ID 3SM9) does contain a chloride ion in the putative chloride binding site. Comparison between the agonist-bound and antagonist-bound structures revealed that the residues comprising this site were identically aligned, and furthermore that the respective water molecule and chloride ion were oriented precisely. To illustrate the proximity of the putative chloride binding site and orthosteric glutamate (Data Supplement 1), Fig. 4A displays the glutamate-bound crystal structure of the mGlu3 receptor ECD (PDB ID 2E4U), where the water molecule (Data Supplement 2) is colored green to represent chloride.
(Ogawa et al., 2010; Acher et al., 2011), our data show that mGlu2 receptors are not activated by chloride. Thus, we hypothesized that the putative chloride binding site present at mGlu3 receptors is disrupted at mGlu2 receptors. To initially evaluate this hypothesis, we generated an in silico model of the mGlu3 receptor ECD (Data Supplement 3) using the Maestro software suite (Schrödinger, 2011a), and built a corresponding homology model of the mGlu2 receptor (Data Supplement 4) using the Prime application (Schrödinger, 2011b) in Maestro. Our mGlu3 receptor model overlaid with the water molecule (colored green to represent chloride) from PDB ID 2E4U shows that chloride is appropriately positioned for hydrogen bonding interactions with the polar hydrogen of the T98 side chain, as well as the polar hydrogens of the Y150 and S149 backbone nitrogens (Fig. 4B). Alternatively, our mGlu2 receptor homology model shows significant differences in the conformations of the corresponding residues (S91, Y144, and S143), all of which are improperly aligned for hydrogen bonding interactions with chloride (Fig. 4C). An overlay of these models (Fig. 4D) suggests that a backbone shift (indicated by arrows) is responsible for these conformational differences, and ultimately for mGlu2 receptor chloride insensitivity.

While the putative chloride binding site is strongly implicated as the orthosteric chloride pocket, the chloride interaction at this site has never been validated empirically. In principle, mutation of T98 to a larger and negatively charged aspartate residue should sterically and electrostatically preclude chloride function. Accordingly, we constructed an mGlu3 receptor T98D mutant, which unlike wild-type mGlu3 receptors showed considerably less LY341495-mediated inverse agonism \( (P = 0.048) \) in the presence of 125 mM chloride, as well as a strong response to glutamate treatment (Fig. 4E). Also in contrast to the wild-type receptor, the mutant receptor responded to both drugs in a chloride-independent manner. This not only demonstrates that T98 is critical for chloride binding, but ultimately supports the hypothesis that the putative chloride binding site is, in fact, the orthosteric binding pocket for chloride. Because T98 is conserved as either a threonine or serine at all group II and III mGlu receptors (Supplemental Fig. 4), it is probable that chloride-mediated activation of mGlu4, mGlu6, and mGlu8 receptors also results from chloride binding in this orthosteric region.

**Chloride Is an Agonist of mGlu3 and mGlu8 Receptors, and an Agonist and Positive Allosteric Modulator of mGlu4 and mGlu6 Receptors.** To investigate the relationship between the two agonists, glutamate and chloride, concentration-response curves of glutamate at several chloride concentrations were generated (Fig. 5, A–D). The parameters \( E_{\text{max}}, E_0, \) and \( EC_{50} \) were analyzed for statistically significant differences (Fig. 5E). The results show no significant difference in the \( E_{\text{max}} \) values of glutamate at mGlu3, mGlu4, mGlu6, or mGlu8 receptors, suggesting that glutamate efficacy is unaffected by chloride. However, the \( E_0 \) values increased in response to increasing concentrations of chloride. These data further support that chloride activates mGlu3, mGlu4, mGlu6, and mGlu8 receptors in the absence of glutamate, and that both ligands are agonists of these receptors. Finally, the \( EC_{50} \) values for glutamate at mGlu4 and mGlu6 receptors were statistically different with increasing chloride, suggesting that chloride acts as a positive allosteric modulator (PAM) of glutamate activity at these receptors. In contrast, the glutamate \( EC_{50} \) values at mGlu3 and mGlu8 receptors appeared to be statistically unaffected (Fig. 5E).

**An Arginine Flip, Elicited by a Single Residue Difference, Dictates the Selectivity of Chloride for mGlu3 versus mGlu2 Receptors.** Next, we sought to understand the structural basis for chloride insensitivity at mGlu2 receptors. Preliminary comparison of our mGlu3 receptor model and corresponding mGlu2 receptor homology model illustrated a change in backbone conformation that appears to preclude chloride binding at mGlu2 receptors (Fig. 4D). Further analysis revealed that this backbone shift stems from a stark conformational difference between corresponding arginine and tyrosine residues, R277/Y150 and R271/Y144, at the mGlu3 and mGlu2 receptor models, respectively (Fig. 6A). The mGlu3 receptor model shows that R277 interacts with Y150, one of the key residues in the orthosteric chloride pocket, via a \( \pi - \text{cation} \) interaction (Fig. 6B). However, at the mGlu2 receptor model, R271 adopts a conformation that precludes a \( \pi - \text{cation} \) interaction (Fig. 6B), causing Y144 to adopt a different orientation that leads to the aforementioned...
backbone shift. Consequently, this arginine flip, which appears to be mediated by two homologous amino acids that are different between mGlu2 and mGlu3 receptors, disrupts the orthosteric chloride site at mGlu2 receptors. While the conformation of R277 at mGlu3 receptors is stabilized by a salt bridge with D279 (E273 at mGlu2 receptors), the orientation of R271 at mGlu2 receptors is stabilized by a salt bridge with D146 (S152 at mGlu3 receptors). Because D146 is a unique residue of mGlu2 receptors (a conserved serine at all other mGlu receptors, see Supplemental Fig. 4), we hypothesized that D146 and S152 are responsible for chloride discrimination between mGlu2 and mGlu3 receptors. To test this hypothesis empirically, an mGlu2 receptor D146S mutant and the corresponding mGlu3 receptor S152D mutant were constructed. In support of our hypothesis, and in contrast to the wild-type mGlu2 receptor (Fig. 7A), the D146S mutant was activated by chloride (Fig. 7C). However, the converse mutation at mGlu3 receptors (S152D) resulted in a chloride-insensitive, constitutively active receptor (Fig. 7D). Based on this unexpected constitutive activity, we broadened our hypothesis to include E273 and D279 as residues responsible for chloride discrimination between mGlu2 and mGlu3 receptors, respectively. Accordingly, a new series of mGlu2 receptor mutants (E273D and D146S/E273D) and corresponding mGlu3 receptor mutants (D279E and S152D/D279E) were constructed. The mGlu2 receptor E273D mutant was chloride insensitive (Supplemental Fig. 2A) and similar to the wild-type mGlu2 receptor. However, the mGlu3 receptor D279E mutant showed a rightward shift in chloride potency (Supplemental Fig. 2B), suggesting that the larger glutamate residue partially destabilizes the $\pi$-cation interaction between Y150 and R277, relative to wild-type mGlu3 receptors. The mGlu2 receptor
D146S/E273D mutant (Supplemental Fig. 2C) showed similar chloride sensitivity to that observed at the mGlu2 receptor D146S mutant, suggesting that E273 does not participate in stabilizing the conformation of R271. Conversely, the mGlu3 receptor S152D/D279E mutant was neither chloride sensitive nor constitutively active (Fig. 7E), effectively resulting in an mGlu3 receptor that behaved like a wild-type mGlu2 receptor. These results demonstrate that the single structural determinant of chloride insensitivity at mGlu2 receptors is D146, which facilitates the R271 arginine flip, thereby precluding a $\pi$-cation interaction with Y144. These findings strongly suggest that the $\pi$-cation interaction between R277 and Y150 structurally rigidifies the orthosteric chloride site at mGlu3 receptors, and that the absence of a similar interaction at mGlu2 receptors prevents chloride-mediated activation.

**Discussion**

Our group and others have reported antagonist-mediated increases in cAMP production at group II and III mGlu receptors (Ma et al., 1997; Suzuki et al., 2007; DiRaddo et al., 2014). In agreement with Suzuki et al. (2007), our data show that antagonist-mediated efficacy is PTX sensitive, indicating an already engaged G$_{ii/o}$-coupled signaling mechanism. Furthermore, we observed LY341495-mediated inverse agonism in primary cultures of cerebellar astrocytes, suggesting that even in the absence of glutamate these receptors are highly active in vivo. Our data demonstrate that high basal group II and III mGlu receptor activity is not due to glutamate contamination, but is rather due to the presence of the endogenous agonist chloride. Before the mGlu receptors were cloned, several studies reported that chloride enhanced $[^{3}H]$glutamate binding in rat brain (Mena et al., 1982), and also in astrocytes at binding sites that were unaffected by kainate and $\text{N}$-methyl-$\text{D}$-aspartate (Bridges et al., 1987). Subsequently, it has been reported that radioligand binding to truncated mGlu3 and mGlu4 receptors was improved by physiologically relevant chloride concentrations (Kuang and Hampson, 2006). In contrast, other studies have suggested that cations affect group II mGlu receptors (Kubo et al., 1998; Schweitzer et al., 2000). Kubo et al. (1998) concluded that increasing concentrations of Ca$^{2+}$ (counterion not specified) activated mGlu3 receptors, but not mGlu2 receptors, although the concurrent increase in chloride concentration was not controlled for. To our knowledge, no functional studies have controlled for chloride concentration while measuring activation of these receptors. Because nearly all physiologic buffers contain chloride, it is likely that chloride has been a confounding variable in many reports of group II and III mGlu receptor activation. While our previous data show that calcium does not modulate glutamate-mediated group II or III mGlu receptor activity (DiRaddo et al., 2014), our current results demonstrate that chloride activates mGlu3, mGlu4, mGlu6, and mGlu8 receptors, but not mGlu2 receptors. Because receptor activation results from direct interaction of the

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**Fig. 5.** Chloride is both an agonist and a positive modulator of glutamate-mediated group II and III mGlu receptor activity. (A–D) Concentration-response curves of glutamate in the presence of increasing chloride concentrations (determined for each receptor based on chloride EC$_{50}$ values from Fig. 3) show an increase in baseline cAMP inhibition ($E_0$) at low glutamate concentrations, but no change in maximal cAMP inhibition ($E_{\text{max}}$) at high glutamate concentrations. The EC$_{50}$ of glutamate was significantly decreased in the absence of chloride at mGlu4 and mGlu6 receptors, but was statistically unaffected by chloride at mGlu3 and mGlu8 receptors. (E) A table of $E_0$, $E_{\text{max}}$, and EC$_{50}$ values highlights statistical differences. All data were normalized as a percent of maximal cAMP levels in the absence of chloride. $E_0$, $E_{\text{max}}$, and EC$_{50}$ values were calculated using the 4-parameter logistic equation. Statistics were calculated using a one-way analysis of variance with a Bonferroni post hoc test, where statistical significance was defined at $P < 0.05$. Data are presented as the mean ± S.E.M. of three individual experiments performed in triplicate. **$P < 0.01$; ***$P < 0.001$. 

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endogenous ion chloride with its own binding site, chloride is by definition an orthosteric agonist of these mGlu receptors. Chloride also potentiates glutamate potency at mGlu4 and mGlu6 receptors, and therefore chloride is a PAM with respect to glutamate activity at these receptors. Future experiments will clarify not only whether glutamate is a PAM with respect to chloride, but also how chloride modulates the potency of other orthosteric ligands. Ultimately, our data show that with the exception of mGlu2 receptors the group II and III mGlu receptors are as much chloride receptors as they are glutamate receptors.

Interestingly, the chloride binding site at group II and III mGlu receptors is structurally conserved at ANP receptors (Acher et al., 2011; Misono et al., 2011). While chloride is not an agonist of ANP receptors, chloride has been shown to modulate ANP receptor oligomerization (Ogawa et al., 2010). In the absence of chloride, ANP receptors are exclusively dimerized, regardless of receptor number. However, in the presence of 150 mM chloride, free monomers predominate but shift toward the dimeric state as a function of increasing receptor expression. Preliminary competition binding experiments revealed complex chloride-dependent effects on both ligand potency and the number of glutamate binding sites at mGlu3 receptors (unpublished data). Further investigation will determine if a similar phenomenon underscores a dynamic interplay between monomeric, homodimeric, and heterodimeric mGlu receptor states, regulated by both chloride concentration and receptor expression.

Because mGlu2 receptors are the only group II and III mGlu receptors that are not activated by chloride, we propose that chloride sensitivity is a fundamental property of these receptors, and that mGlu2 receptor chloride insensitivity is exceptional. Our results suggest that this chloride insensitivity stems from the absence of a $\sigma$-cation interaction between R271 and Y144, which is present between R277 and Y150 at mGlu3 receptors. While this $\sigma$-cation interaction has been previously described as a structural motif of both mGlu2 and mGlu3 receptors (Muto et al., 2007; Lundström et al., 2009), it is not absolutely required for glutamate-mediated activation (Malherbe et al., 2001; Yao et al., 2003; Lundström et al., 2009), and our data support that it is only present at mGlu3 receptors. Instead, we propose that D146 at mGlu2 receptors (a conserved serine at all other mGlu receptors) precludes a $\sigma$-cation interaction by facilitating an arginine flip, relative to mGlu3 receptors, which consequently disrupts the orthosteric chloride site. Supporting this claim is the chloride-sensitive mGlu2 receptor D146S and D146S/E273D mutants and the chloride-insensitive mGlu3 receptor S152D and S152D/D279E mutants. This swap of chloride sensitivity by site-directed mutagenesis is consistent with other studies, where the differential ion sensitivities of the wild-type receptors were reversed by D146S mutation at mGlu2 receptors and the corresponding S152D mutation at mGlu3 receptors (Kubo et al., 1998; Malherbe et al., 2001). We assert that the arginine flip is induced by mutation of a single amino acid, which acts as a chloride switch between mGlu2 and mGlu3 receptors. Because the arginine and tyrosine residues are not conserved through the group III mGlu receptors (Supplemental Fig. 4), this chloride switch marks a unique structural divergence between mGlu3 and mGlu2 receptors, which share 80% sequence homology and are largely thought to activate and signal in the same fashion.

Despite a shared $G_{\text{i/o}}$-coupled signaling mechanism, recent studies demonstrate that activation of mGlu2 and mGlu3 receptors can elicit opposing neurotrophic and cognitive effects (Taylor et al., 2002, 2005; Corti et al., 2007; Fell et al., 2008; Caraci et al., 2012). This highlights the need for subtype-selective agonists to evaluate the therapeutic potential of each mGlu receptor. Unfortunately, selective activation of mGlu3 or mGlu2 receptors has been difficult to achieve due to the lack of identified structural differences. Our results demonstrate a subtle difference in tyrosine conformation that may be exploitable for drug selectivity. In support of this
exception of mGlu2 receptors, the group II and III mGlu receptors share the putative chloride binding site (Acher et al., 2011), although mGlu2 receptors, respectively. Because models do not predict different conformations of S278 and S272, the selective mGlu2 receptor agonist may actually exploit different conformations of the neighboring tyrosine and/or arginine residues.

Our results also reveal a second region of dissimilarity between mGlu3 and mGlu2 receptors: the orthosteric chloride site. While targeting this region has already been proposed as a strategy for the design of subtype-selective group III mGlu receptor agonists (Acher et al., 2011; Goudet et al., 2012), mGlu4, mGlu6, and mGlu8 receptors all contain functional chloride sites. In contrast to the claim that all mGlu receptors share the putative chloride binding site (Acher et al., 2011), our results demonstrate that this site is not conserved at mGlu2 receptors. Thus, the opportunity for subtype selectivity between group II mGlu receptors by targeting the orthosteric chloride site (or lack thereof) is much more pronounced than for group III mGlu receptors, where this strategy may have already had some success (Goudet et al., 2012). The close proximity of the chloride pocket and glutamate binding site, as well as the effects of chloride on glutamate potency at mGlu4 and mGlu6 receptors, renders selective orthosteric drug design inextricably intertwined with chloride function.

Chloride, an endogenous anion generally deemed a passive participant in neuronal excitability, is an agonist of mGlu3 and mGlu8 receptors and an antagonist and PAM of mGlu4 and mGlu6 receptors that acts as its own orthosteric pocket. Therefore, we conclude that these mGlu receptors have two distinct orthosteric sites (Christopoulos et al., 2014), one for glutamate and one for chloride. Our data show that, with the exception of mGlu2 receptors, the group II and III mGlu receptors are as much chloride receptors as they are glutamate receptors. Furthermore, chloride insensitivity of mGlu2 receptors results from a single amino acid difference, highlighting a molecular switch for chloride insensitivity that may be transduced through an arginine flip. These structural differences between mGlu2 and mGlu3 receptors mark a new strategy for the design of subtype-selective group II mGlu receptor agonists, which must be considered within the context of chloride function. Ultimately, our findings underscore a scenario in which either (1) mGlu3, mGlu4, mGlu6, and mGlu8 receptors are highly active in vivo due to static extracellular chloride concentrations; or (2) as can be deduced from recent literature (Glykys et al., 2014), chloride microdomains exist throughout the central nervous system, rendering local extracellular chloride levels far more tightly regulated, both spatially and temporally, than currently accepted.

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Authorship Contributions

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References


Chloride is an Agonist of Group II and III mGlu Receptors


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