Allosteric modulation of metabotropic glutamate receptors by chloride ions

Amélie S. Tora,∗† Xavier Rovira,∗† Ibrahima Dione,‡ Hugues-Olivier Bertrand,§ Isabelle Brabet,*† Yves De Koninck,‡ Nicolas Doyon,‡ Jean-Philippe Pin,∗† Francine Acher,∗† and Cyril Goudet∗†,1

*Institut de Génomique Fonctionnelle, CNRS, UMR 5203, Université de Montpellier, Montpellier, France; †INSERM U1191, Montpellier, France; ‡Centre de Recherche de l’Institut Universitaire en Santé Mentale du Québec and Université Laval, Québec, Canada; §Biovia Dassault Systèmes, Orsay, France; and §Laboratoire de Chimie et Biochimie Pharmacologiques et Toxicologiques, CNRS, UMR 8601, Université Paris Descartes, Sorbonne Paris Cité, Paris, France

ABSTRACT Metabotropic glutamate receptors (mGluRs) play key roles in the modulation of many synapses. Chloride (Cl\(^-\)) is known to directly bind and regulate the function of different actors of neuronal activity, and several studies have pointed to the possible modulation of mGluRs by Cl\(^-\). Herein, we demonstrate that Cl\(^-\) behaves as a positive allosteric modulator of mGluRs. For example, whereas glutamate potency was 3.08 ± 0.33 μM on metabotropic glutamate (mGlu) 4 receptors in high-Cl\(^-\) concentration, signaling activity was almost abolished in low Cl\(^-\) in cell-based assays. Cl\(^-\) potency was 78.6 ± 3.5 mM. Cl\(^-\) possesses a high positive cooperativity with glutamate (Hill slope ≈ 6 on mGlu4), meaning that small variations in [Cl\(^-\)] lead to large variations in glutamate action. Using molecular modeling and mutagenesis, we have identified 2 well-conserved Cl\(^-\) binding pockets in the extracellular domain of mGluRs. Moreover, modeling of activity-dependent Cl\(^-\) variations at GABAAergic synapses suggests that these variations may be compatible with a dynamic modulation of the most sensitive mGluRs present in these synapses. Taken together, these data reveal a necessary role of Cl\(^-\) for the glutamate activation of many mGluRs. Exploiting Cl\(^-\) binding pockets may yield to the development of innovative regulators of mGluR activity.—Tora, A. S., Rovira, X., Dione, I., Bertrand, H.-O., Brabet, I., De Koninck, Y., Doyon, N., Pin, J.-P., Acher, F., Goudet, C. Allosteric modulation of metabotropic glutamate receptors by chloride ions. FASEB J. 29, 000–000 (2015). www.fasebj.org

Key Words: allostery · GABA · GPCR · PAM · anion

Chloride (Cl\(^-\)) is the most abundant physiologic anion. The importance of Cl\(^-\) is highlighted by the diverse human diseases associated with an altered Cl\(^-\) homeostasis, including cystic fibrosis, hypertension, epilepsy, and neuropathic pain (1). Indeed, Cl\(^-\) is involved in many cellular functions, including volume control, intracellular membrane trafficking, and excitability. Not only does Cl\(^-\) flow through GABAA and glycine ligand-gated channels that mediate GABA and glycine activity at inhibitory synapses, but Cl\(^-\) is also known to bind and regulate the function of different actors of neuronal activity, such as ion channels (2), transporters (3–5), and receptors (6).

The GPCRs activated by the neurotransmitter glutamate, also called metabotropic glutamate receptors (mGluRs), are key modulators of neurotransmission in the CNS (7, 8). The mGluR family is composed of 8 members [metabotropic glutamate (mGlu) 1–8] and is divided into 3 groups. Group I mGluRs (mGlu1 and 5) are postsynaptic receptors that positively modulate neuronal excitability. Group II (mGlu2–3) and most of group III (mGlu4, 7, and 8) are mainly presynaptic receptors expressed both in glutamatergic and GABAAergic synapses where they decrease neurotransmission. The other group III receptor, mGlu6, is a postsynaptic receptor mostly expressed in bipolar ON cells in the retina (9). Structurally, these receptors are composed of 3 juxtaposed domains: a core 7 transmembrane-spanning domain, common to all GPCRs, is linked via a rigid cysteine-rich domain to the Venus flytrap (VFT), a large bilobed extracellular domain where glutamate binds (10). Besides the well-documented regulation of mGluRs by extracellular calcium (Ca\(^{2+}\)) and gadolinium (Gd\(^{3+}\)) (11–13), several studies have pointed to the Cl\(^-\) dependence of glutamate binding on mGluRs (14–17). However, the molecular basis and the putative physiopathologic relevance of this dependence remain unknown.

Abbreviations: AVP, arginine vasopressin peptide; AVPR, arginine vasopressin peptide receptor; CaSR, calcium-sensing receptor; [Cl\(^-\)], chloride concentration; [CI\(^-\)], intracellular chloride concentration; FRET, fluorescence resonance energy transfer; GPI, glycosyl phosphatidylinositol; HA, human influenza hemagglutinin; (continued on next page)
Here, we demonstrate that extracellular Cl\(^-\) behaves like a positive allosteric modulator (PAM) of the different members of the mGluR family. Cl\(^-\) dramatically favors the agonist-induced active conformation of the receptors, as demonstrated through the use of mGlu fluorescence resonance energy transfer (FRET)-based biosensors. Using molecular modeling and site-directed mutagenesis, we have identified critical residues in the extracellular domain of mGlu4 and mGlu2, which are involved in Cl\(^-\) sensitivity. Moreover, electrodiffusion modeling suggests that variations of extracellular Cl\(^-\) occurring in the vicinity of the synaptic cleft of a GABAergic synapse during activation may be compatible with a dynamic modulation of mGluR function.

MATERIALS AND METHODS

Cell transfection

Human embryonic kidney (HEK) 293 cells were transiently transfected with rat mGluR receptors by electroporation and seeded in a polyornithine-coated 96-well plate at the density of 150,000 cells well. 5HT2b and oxytocin (OT) receptors were used as controls. Group II and III receptors were cotransfected with a chimeric G\(_\alpha\)/G\(_\beta\) protein (G\(_\beta\)TOP) to allow measurements of Ca\(^{2+}\) mobilization. All mGluRs were also cotransfected with EAAC1, a glutamate transporter, to avoid influence of extracellular glutamate. Cells were cultured in DMEM (Invitrogen, Life Technologies) to reduce extracellular glutamate. Cells were cultured in DMEM (Invitrogen, Life Technologies, Cergy Pontoise, France), supplemented with 10% fetal bovine serum. Medium was changed by GlutaMAX (Invitrogen, Life Technologies, Cergy Pontoise, France), supplemented with 10% fetal bovine serum. Medium was changed by GlutaMAX (Invitrogen, Life Technologies) to reduce extracellular glutamate concentration 3 h before stimulation.

Chloride buffers composition

All chemical products were purchased from Sigma-Aldrich (L’Isle d’Abeau, France). Full Cl\(^-\) buffer contained 154.6 mM Cl\(^-\) ions and was the reference buffer (referred to as high-Cl\(^-\) buffer) used for Ca\(^{2+}\) signaling and conformational sensor assay. It included 150 mM NaCl, 2.6 mM KCl, 1.18 mM MgSO\(_4\), 10 mM d-glucose, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 1 mM Ca\(_{123}\), and 0.5% (w/v) bovine serum albumin. For buffers with lower [Cl\(^-\)], NaCl and KCl were progressively replaced by glutamate transporters (NaC\(_{6}\)H\(_{11}\)O\(_{7}\) and KC\(_{6}\)H\(_{11}\)O\(_{7}\), respectively). pH was adjusted at 7.4.

Calcium mobilization assay

For this assay, black-walled, clear-bottom 96-well plates were used. At 24 h after transfection, cells were loaded with 1 mM calcium-sensitive fluorescent dye (Fluo-4 AM; Invitrogen, Life Technologies) diluted in fresh Cl\(^-\) buffer (154.6 mM) for 1 h at 37\(^\circ\)C. Then, cells were washed and maintained in buffers with [Cl\(^-\)] ranging from 2 to 154.6 mM supplemented with 4 mM probenecid. Agonists were also diluted in appropriate buffers. Ca\(^{2+}\) release was determined using FlexStation 3 (Molecular Devices, Sunnyvale, CA, USA). Fluorescence was detected for 60 s at 485 nm excitation and 525 nm emission. Calcium mobilization was also quantified after stimulation of an arginine vasopressin peptide (AVP)-sensitive endogenous receptor [arginine vasopressin peptide receptor (AVPR)] on nontransfected cells in high and low Cl\(^-\) in order to assess glutamate side effects on cell signaling. This was set as an internal control in each experiment.

Conformational sensor assay

Flag-SNAP-tagged human mGluRs were used for this assay. Cells were seeded in black-walled, black-bottom 96-well plates. At 24 h after transfection, cells were labeled with 100 nM SNAP-Lumi4-Tb and 60 nM SNAP-Green diluted in 154.6 mM Cl\(^-\) buffer. After 1 h at 37\(^\circ\)C, cells were washed in appropriate Cl\(^-\) buffers, and drugs were added. Time-resolved FRET measurements were collected at 520 nm using the PHERAstar F5 microplate reader as previously described (18).

Molecular modeling of chloride binding sites

Calculations were carried out in Discovery Studio 4.0 (Biovia, Dassault Systèmes, San Diego, CA, USA). Homology models of the amino terminal domains of mGlu4 were built using mGlu1 and mGlu3 amino terminal domains as templates [Protein Data Bank (PDB) codes 1EWK and 2E4U, respectively], according to the previously described methodology (19). Homology models of the amino terminal domains of mGlu2 were built using mGlu3 amino terminal domain as a template (PDB code 2E4U). A multiple sequence alignment of all the mGluR amino terminal domain sequences was generated and was subsequently used to generate 100 models using Modeler (20). The 10 best models according to Modeler’s probability density function were selected and further evaluated using Profiles 3D (21) and Ramachandran violations. The final model exhibits the highest Profiles 3D score and the lowest number of Ramachandran violations. Chloride ions were then positioned in our models by lobe superimposition with mGlu3 amino terminal domain (PDB code 3SM9). It is worth noting that although 3SM9 exhibits 2 Cl\(^-\) binding sites, it was not used for comparative modeling because it is an open form structure. Models were eventually refined by minimization using Chemistry at Harvard Macromolecular Mechanics (22).

Site-directed mutagenesis

Mutant receptors were obtained using the QuikChange Site-Directed Mutagenesis strategy (Strategene, La Jolla, CA, USA). All mutations were made in the human influenza hemagglutinin (HA) SNAP-tagged rat mGlu4- or mGlu2-encoding plasmids and were verified by sequencing. Total and cell surface expression was quantified by ELISA using anti-HA primary antibody coupled to peroxidase (3F10; Roche, Basel, Switzerland) as previously described (23).

Data analysis

Data from calcium mobilization and conformational sensor assays were analyzed with Prism 6 software (GraphPad Software, San Diego, CA, USA). In each experiment, a 4-parameter concentration-response curve equation was used to fit data, and potency (EC\(_{50}\)) was estimated as logarithms (log EC\(_{50}\)). For clarity, absolute (positive) logarithms (pEC\(_{50}\)) were used. E\(_{\text{max}}\) represents the maximum response obtained at saturating agonist concentrations.

(continued from previous page)

HEK, human embryonic kidney; mGlu, metabotropic glutamate; mGluR, metabotropic glutamate receptor; NFR, natriuretic peptide receptor; OT, oxytocin; PAM, positive allosteric modulator; PDB, Protein Data Bank; TESA, T108E plus S160A; TESD, T108E plus S160D; VFT, Venus flytrap; WT, wild-type.
concentration, and nH (Hill slope) describes the steepness of the curve. Data shown in the figures represent the means ± SEM of at least 3 experiments.

Raw data were normalized according to values obtained in the high-Cl\textsuperscript{-} buffer. This buffer was used in routine pharmacologic experiments and was set as reference buffer. In calcium mobilization assays in Fig. 1, relative fluorescence units were normalized to the maximum response observed in high-Cl\textsuperscript{-} buffer, which corresponds to the top asymptote of the fitting curve. When a full concentration-response curve was not measurable within the concentration range tested in low-Cl\textsuperscript{-} buffer, the maximum response was arbitrarily set at 100% to extrapolate the pEC\textsubscript{50}. Data obtained in increased chloride concentration ([Cl\textsuperscript{-}]) buffers were normalized to each data set basal response (bottom asymptote, vehicle addition) and to the maximum response observed in high-Cl\textsuperscript{-} reference buffer. Functional data of mutants were also normalized to the baseline (bottom asymptote, vehicle addition) and to wild-type (WT) maximum response in high-Cl\textsuperscript{-} buffer. Chloride sensitivity of each construct is represented by the loss of agonist potency due to chloride depletion and is calculated by the difference (ΔpEC\textsubscript{50}) between pEC\textsubscript{50} in low- and high-Cl\textsuperscript{-} buffers. Statistical differences between ΔpEC\textsubscript{50} and pEC\textsubscript{50} were determined by 1-way ANOVA with Dunnett's post-test. In conformational sensor assays (Fig. 2), high FRET ratio measured in each data set for basal condition (vehicle addition) and low FRET ratio obtained in saturating agonist concentration in the high-Cl\textsuperscript{-} buffer were used to define respectively 100 and 0% of the signal as indicated in Fig. 2A.

Electrodiffusion computational modeling

To simulate fluctuations of [Cl\textsuperscript{-}] within the synaptic cleft as well as in the convoluted extracellular space surrounding it, we solved the Poisson-Nernst-Planck equations (24) using the method of finite elements (25). This approach relies on a discretization of the modeled space with a tetrahedral mesh and describes the dynamic evolution of ionic concentrations and electric potential at each node of the mesh and at each time step. Our mesh was composed of 178,000 tetrahedrons. The extracellular space consists of thin layers of interstitial medium separating neurons and glial cells, so that the synaptic cleft and its surroundings are mostly sealed from the chemical standpoint. The width of the thin sheets composing the interstitial extracellular space has been measured in the range of a few tens of nanometers (10–40 nm) (26–28). In choosing our model geometry, we aimed to capture the hindrance of diffusion in the perisynaptic extracellular space (29) and the relative compartmentalization of the extracellular space (30), but it is impossible to provide a stereotypic description of the interstitial space given that it exhibits great variability (26). In our model geometry, the width of the synaptic cleft as well as the distance between the presynaptic terminal and the glial cells or between glial cells themselves were chosen to be

Figure 1. mGluRs are sensitive to extracellular chloride. Calcium mobilization assays performed in HEK293 cells transiently expressing rat mGluRs (A–F) and human 5HT2b and OT receptors (G and H, respectively). A chimeric G protein was cotransfected if necessary to allow measurement of intracellular calcium release. Calcium signaling of an AVP-sensitive endogenous receptor (AVPR) was also quantified on nontransfected cells (I). Depletion in [Cl\textsuperscript{-}] (154.6 mM; solid circles) to low Cl\textsuperscript{-} (2 mM; open circles) significantly reduces mGluR signaling in contrast to the Cl\textsuperscript{-}-insensitive receptors 5HT2b, OT, and endogenous AVPR. Quantification of loss of potency due to Cl\textsuperscript{-} depletion (ΔpEC\textsubscript{50}) (J) shows that mGlu2 is the least sensitive to Cl\textsuperscript{-} depletion (1-way ANOVA with Dunnett’s posttest value of P < 0.05, compared with 5HT2b); mGlu6, mGlu1, and mGlu5 display an intermediate sensitivity (P < 0.001), whereas mGlu4 and 8 are the most sensitive to extracellular Cl\textsuperscript{-} (P < 0.001). mGlu4 and mGlu8 pEC\textsubscript{50} values in low-Cl\textsuperscript{-} buffer were calculated from extrapolated curves where the maximum response was arbitrarily set at 100%. Relative fluorescence units were normalized by the maximum response (top asymptote) of the receptor in the high-Cl\textsuperscript{-} buffer in each experiment. Concentration-response curve parameters are listed in Supplemental Table S1. VEH, effect of free-agonist buffer (vehicle) on Ca\textsuperscript{2+} mobilization. *P < 0.05; ***P < 0.001.

ALLOSTERIC MODULATION OF MGLURS BY Cl\textsuperscript{-}
midrange: 20 nm (31). The width of the membrane and synapse was set at 7 nm. The diameter of the synapse was set at 200 nm, whereas that of the presynaptic terminal was 400 nm (31). The length of the presynaptic terminal was set to 2 μm by which the boundary of the domain is reached.

The synaptic GABA_A permittivity to Cl^- was chosen so that the maximal synaptic conductance during a single GABA_A event was 2 nano Siemens (nS), where conductance was converted into permittivity permeability through the Goldman-Hodgkin-Katz equation. The time course of GABA_A conductance was described by an α function corresponding to a rise time of 1 ms and a decay time of 20 ms (32). The initial value of extracellular chloride concentration ([Cl^-]_o) was 120 mM. To model the impact of a Cl^- influx (a hyperpolarizing GABA_A event), we used an initial intracellular chloride concentration ([Cl^-]_i) of 5 mM, whereas to model Cl^- efflux, this initial value was 35 mM (33). For the simulation of a single hyperpolarizing GABA_A event, we assumed a holding membrane potential of −40 mV, a realistic value under the assumption that concurrent depolarizing activity (e.g., action potential firing or glutamatergic inputs) occurs in the vicinity of the GABA_A synapse, which is likely to happen in high-conductance states (34). For the simulation of a single depolarizing GABA_A event, we assumed a holding membrane potential of −65 mV. In both cases, we used constant concentration boundary conditions, which physically correspond to the modeled geometry being linked to an infinite bath with constant Cl^- concentration. We also modeled the [Cl^-]_i response to a 200 ms train of 20 Hz GABA_A events occurring jointly with repeated excitatory activity; in which instance, we updated dynamically the membrane potential. Accounting for the fact that, in the repeated activity scenario, Cl^- accumulation may reach beyond the geometry described by our tetrahedral mesh, we assumed the modeled geometry to be surrounded by a shell of Frankenhaeuser Hodgkin space connected to an infinite bath with constant ionic concentration (35, 36). In the latter case, we used additional Matlab (MathWorks, Natick, MA, USA) codes to compute boundary conditions of Cl^- concentration at the interface of the Frankenhaeuser Hodgkin space as well as to compute the boundary condition of electric potential defined at the edge of the modeled membrane.

Figure 2. Chloride favors the agonist-induced active conformation of mGluR VFT via an action on the extracellular domain of the receptors. A) Schematic representation of FRET-based full-length mGlu sensor. Conformational sensor assays performed in HEK293 cells transiently expressing human mGlu2, 1, 5, and 4 sensors (B–E). A depletion in [Cl^-]_o from high Cl^- (154.6 mM; solid circles) to low Cl^- (2 mM; open circles) significantly reduces mGluR conformational change measurements. F) Schematic representation of truncated FRET-based mGlu sensor. The truncated construct corresponds to the receptor’s VFT alone with a GPI anchor to allow membrane expression (VFT-GPI). Quantification of loss of potency due to Cl^- depletion (ΔpEC_{50}) (G) correlates to functional data in Fig. 1. Conformational sensor assays performed in HEK293 cells transiently expressing human mGlu2 (H) or mGlu4 (I) full-length receptor (circles) or truncated (squares). Similar results between full-length and GPI constructs in high- (solid shape) and low-Cl^- buffer (open shape) experiments suggest that Cl^- sensitivity is carried by the receptor’s VFT. Data were normalized by the basal (100% FRET ratio) and high-Cl^- buffer maximum response (0% FRET ratio). Concentration-response curve parameters are listed in Supplemental Table S1. VEH, effect of free-agonist buffer (vehicle) on Ca^{2+} mobilization.
RESULTS

mGluRs are sensitive to extracellular chloride

We first evaluated Cl\(^-\) sensitivity of mGluRs by quantifying their Ca\(^{2+}\) signaling activity upon agonist binding in HEK293 cells transiently expressing mGlu1–8 receptors (Fig. 1). To allow the measurement of intracellular Ca\(^{2+}\) release, receptors usually coupled to G\(_i\) protein were cotransfected with a modified G\(_i\) protein (G\(_3\)TOP) that allows G\(_i\)-coupled mGluRs to activate PLC (37). Ca\(^{2+}\) mobilization assays were performed in buffers containing 154.6 or 2 mM Cl\(^-\). These will be referred to as high-Cl\(^-\) and low-Cl\(^-\) buffers, respectively. In the low-Cl\(^-\) buffer, chloride chemical species were replaced by gluconate equivalents to maintain the osmolarity of the medium.

To assess potential effects of gluconate replacement on cell capacity to mobilize Ca\(^{2+}\), signaling activity of an AVP-sensitive endogenous receptor (AVPR) was quantified on nontransfected cells. In low-Cl\(^-\) buffer, endogenous AVPR signaling is not changed, indicating that gluconate species do not alter cell capacity to mobilize Ca\(^{2+}\) because endogenous AVPR signaling is not changed (Fig. 1I). Depletion of [Cl\(^-\)]\(_m\) results in a specific decrease of all mGluR signaling activity (Fig. 1A–F) in contrast to other GPCRs such as the serotonin (5HT2b) (Fig. 1G) and OT receptors (Fig. 1H). \(\Delta\)pEC\(_{50}\) measurements representing the loss of agonist potency due to chloride depletion were used to characterize Cl\(^-\) sensitivity of the receptors (Fig. 1/ and Supplemental Table S1). mGlu2 is the least sensitive to Cl\(^-\) (Fig. 1A), showing a 0.54 ± 0.06 log decrease in glutamate pEC\(_{50}\); mGlu6, 1, and 5 exhibit an intermediate sensitivity with a decrease of 0.94 ± 0.09, 1.54 ± 0.11, and 1.68 ± 0.05 glutamate pEC\(_{50}\), respectively (Fig. 1B–E). mGlu8 and mGlu4 receptors are the most sensitive ones because Cl\(^-\) depletion abolished Ca\(^{2+}\) signaling within the range of glutamate concentrations tested, except for the highest, 1 mM (Fig. 1E, F). Their putative pEC\(_{50}\) values in low-Cl\(^-\) buffer were estimated from extrapolated curves, and \(\Delta\)pEC\(_{50}\) values were calculated. Thus, Cl\(^-\) sensitivity was extrapolated to a loss in glutamate pEC\(_{50}\) of 2.61 ± 0.13 log for mGlu8 and 2.8 ± 0.16 log for mGlu4 (Fig. 1F). However, more potent agonists (L-AP4 and LSP4-2022) rescue their signaling responses in low-Cl\(^-\) buffer and reduce the loss of potency observed with Cl\(^-\) depletion. Interestingly, mGlu4 signaling activity with LSP4-2022 showed a significantly reduced Cl\(^-\) sensitivity compared with LAP4 (P < 0.05) (Supplemental Table S1). A signaling response was not measurable for mGlu7 with glutamate because of its low affinity for this ligand. However, concentration-response curves were obtained with LAP4, showing that mGlu7 Cl\(^-\) sensitivity is similar to mGlu4 and mGlu8 (Supplemental Table S1).

Chloride sensitivity is carried by the extracellular domain of mGluRs

Considering the results of Ca\(^{2+}\) and conformational sensor assays, we decided to focus on understanding the difference between the least and the most sensitive receptors to extracellular Cl\(^-\): mGlu2 and mGlu4. To determine which domain of the receptor is involved in Cl\(^-\) sensitivity, we used truncated versions of the sensors, which lack the transmembrane domain. We measured the influence of Cl\(^-\) on the conformational dynamics of the extracellular domain of mGlu2 or mGlu4 attached to the plasma membrane through a glycosyl phosphatidylinositol (GPI) anchor (VFT–GPI mGlu2 and VFT–GPI mGlu4) (Fig. 2F).

Interestingly, as previously shown (18), such truncated sensors behave like the full-length mGlu2 or mGlu4 sensor with similar basal and glutamate-induced FRET signals and with the same glutamate potencies. Moreover, VFT–GPI mGlu2 and VFT–GPI mGlu4 showed a highly similar profile regarding Cl\(^-\) depletion compared with full-length receptors, suggesting that Cl\(^-\) sensitivity is carried by the VFT (Fig. 2H, I).

Molecular basis of mGlu4 and mGlu2 differential chloride sensitivity

Molecular modeling of mGlu2 and mGlu4 extracellular domains was performed to assess Cl\(^-\) binding. Our homology models suggest the presence of 2 functional Cl\(^-\) binding sites in mGlu4 (Fig. 3A) and only 1 site in mGlu2 (Fig. 3D). In mGlu4, a first site is located in lobe 1, and the Cl\(^-\) ion appears to be embedded in the structure. This site will be further referred to as site 1. Cl\(^-\) is bound to the side chain of Thr108 and to the backbone (NH) of Ser157 and Gly158 through hydrogen bonds (Fig. 3B). This site is also

ALLOSTERIC MODULATION OF MGLURS BY Cl\(^-\)

surrounded by Phe56, Ser110, and Val161 (Supplemental Fig. S1 A). The second site is located in lobe 2 in the open “resting” conformation of the VFT and interacts with both lobes in the closed “active” conformation. This site will be further referred to as site 2. In mGlu4, Cl\textsuperscript{−} is bound to Ser157 through hydrogen bonds. Site 2 is located at the interface of the 2 lobes where the Cl\textsuperscript{−} is bound to Ser160 (lobe 1) and Asn286 (lobe 2) (B). C) Multiple sequence alignment of the 8 mGluRs. Thr108 and Ser160 residues putatively involved in Cl\textsuperscript{−} binding are conserved among all 8 mGluRs except in mGlu2 where the serine in site 2 is replaced by Asp146. Alignment performed with MUSCLE (MUltiple Sequence Comparison by Log-Expectation) software (European Molecular Biology Laboratory-European Bioinformatics Institute) using sequence accession numbers NP_058707.1, NP_058708.1, NP_075209.1, NP_112302.1, NP_073157.1, NP_071538.1, NP_001099181.1, and NP_001099182.1 and annotated with Jalview. Purple color intensity is correlated with the percentage of amino acid conservation. Numbers correspond to mGlu4 amino acid numbering. D) Molecular modeling of mGlu2 extracellular domain regarding mGlu3 crystal structure and mGluR alignment. The Cl\textsuperscript{−} ion in site 1 is bound to the side chain of Ser91 and to the backbone NH of Ser143. The carboxylate moiety of Asp146 mimics Cl\textsuperscript{−} presence and is positioned by a hydrogen bond with the backbone NH of Tyr216 and a salt bridge with Arg271.

Figure 3. Molecular modeling of chloride binding sites in mGlu4 and mGlu2 extracellular domains (VFT). A) Molecular modeling of mGlu4 VFT based on the crystal structure of mGlu1 and mGlu3 VFTs (PDB 1EWK and 2E4U, respectively). Cl\textsuperscript{−} binding site 1 is located in lobe 1. It contains a Cl\textsuperscript{−} bound to the side chain of Thr108 and the backbone NH of Ser157 and Gly158 through hydrogen bonds. Site 2 is located at the interface of the 2 lobes where the Cl\textsuperscript{−} is bound to Ser160 (lobe 1) and Asn286 (lobe 2) (B). C) Multiple sequence alignment of the 8 mGluRs. Thr108 and Ser160 residues putatively involved in Cl\textsuperscript{−} binding are conserved among all 8 mGluRs except in mGlu2 where the serine in site 2 is replaced by Asp146. Alignment performed with MUSCLE (MUltiple Sequence Comparison by Log-Expectation) software (European Molecular Biology Laboratory-European Bioinformatics Institute) using sequence accession numbers NP_058707.1, NP_058708.1, NP_075209.1, NP_112302.1, NP_073157.1, NP_071538.1, NP_001099181.1, and NP_001099182.1 and annotated with Jalview. Purple color intensity is correlated with the percentage of amino acid conservation. Numbers correspond to mGlu4 amino acid numbering. D) Molecular modeling of mGlu2 extracellular domain regarding mGlu3 crystal structure and mGluR alignment. The Cl\textsuperscript{−} ion in site 1 is bound to the side chain of Ser91 and to the backbone NH of Ser143. The carboxylate moiety of Asp146 mimics Cl\textsuperscript{−} presence and is positioned by a hydrogen bond with the backbone NH of Tyr216 and a salt bridge with Arg271.

An alignment of the residues putatively involved in Cl\textsuperscript{−} binding is displayed in Fig. 3C. In site 1, only Val161 is conserved in all mGluRs, whereas a polar serine or threonine residue is always found at Thr108 position in mGlu4. Group I receptors (mGlu1 and 5) show the most divergent sequences (Supplemental Fig. S1B). Phe56 and Ser110 are conserved in groups II and III. Ser157 is also conserved within these groups, except in mGlu8, where it is replaced by an alanine. Regarding site 2, alignment shows the conservation of a signature motif composed of amino acids Ser159 and Tyr230. Interestingly, Ser160 is conserved in all mGluRs except in mGlu2, where the serine is replaced by an aspartate (Asp146). Indeed, mGlu2 VFT is compatible with the presence of only 1 Cl\textsuperscript{−} binding site in lobe 1 (site 1), where Ser91, equivalent of Thr108 in mGlu4, coordinates the Cl\textsuperscript{−} ion. In mGlu2 then, site 2 is occupied by the negatively charged carboxylate of Asp146 that likely mimics Cl\textsuperscript{−} presence and makes hydrogen bonds with the NH backbone of Tyr216 and a salt bridge with Arg271 (Fig. 3D). We speculate that the lack of site 2 in mGlu2 could be responsible for the difference in Cl\textsuperscript{−} sensitivity between mGlu4 and mGlu2.

**Determination of the chloride binding site in mGlu2**

The molecular basis of mGlu2 Cl\textsuperscript{−} sensitivity was delineated by site-directed mutagenesis, on the basis of molecular modeling and sequence alignments (Fig. 4).
Mutation of Ser91 into an alanine (S91A) significantly reduced mGlu-mediated \( \text{Ca}^{2+} \) signals both in high- and low-Cl\(^-\) buffers compared with the WT (Fig. 4A, B). S91A seemed to be more sensitive to Cl\(^-\) depletion, suggesting that this mutation weakens Cl\(^-\) binding. To prevent Cl\(^-\) binding, Ser91 had to be replaced by a glutamate (S91E), which mimics Cl\(^-\) thanks to its carboxylate moiety (Fig. 4C). The functional response was almost fully rescued, and the receptor was insensitive to Cl\(^-\) depletion compared with the WT \((P < 0.05)\) (Fig. 4E and Supplemental Table S2). To recreate the second Cl\(^-\) binding site, we performed a D146S mutation, which introduced a serine amino acid, allowing Cl\(^-\) to bind through its hydroxyl side chain (Fig. 4D). In high-Cl\(^-\) buffer, the WT receptor and mGlu2 D146S responded similarly to glutamate. However, the D146S mutant was more sensitive to Cl\(^-\), displaying a glutamate \( \Delta \text{pEC}_{50} \) of -1.83 ± 0.12 compared with the WT \((P < 0.001)\) (Fig. 4E and Supplemental Table S2). No significant variations were found in the expression profiles of any of these constructs (Supplemental Fig. S1C).

Our models, in conjunction with our experimental data, suggest that mGlu2 Cl\(^-\) sensitivity is carried mainly by Ser91 in lobe 1, and Asp146 mimics Cl\(^-\) at the interface of the 2 lobes. Taken together, these results demonstrate that mGlu2 has only 1 functional Cl\(^-\) binding site.

**Determination of the chloride binding sites in mGlu4**

A similar strategy was used to determine the molecular basis of mGlu4 Cl\(^-\) sensitivity (Fig. 5). Based on molecular modeling, all amino acids surrounding the 2 putative Cl\(^-\) binding sites were mutated into an alanine by site-directed mutagenesis (Supplemental Table S2). L-AP4 was used in the \( \text{Ca}^{2+} \) mobilization assay because this ligand rescues a functional response in low-Cl\(^-\) buffer, allowing Cl\(^-\) sensitivity measurements by \( \Delta \text{pEC}_{50} \). In site 1, the T108A mutation almost abolished \( \text{Ca}^{2+} \) signaling of the receptor in both high- and low-Cl\(^-\) buffers compared with the WT (Fig. 5A, B). Mimicking chloride binding by replacing this amino acid by a glutamate (T108E) partially rescued a functional response in both buffers (Fig. 5C). The mutated receptor was still sensitive to Cl\(^-\) depletion, similarly to the WT receptor, probably due to the second Cl\(^-\) binding site. Alanine replacement also revealed a role of Phe56 and Val161 in mGlu4 Cl\(^-\) sensitivity (Supplemental Table S2). The P56A mutation did not alter L-AP4 \( \text{pEC}_{50} \) in high-Cl\(^-\) buffer but decreased it in the low-Cl\(^-\) condition. On the contrary, V161A mutant displayed an enhanced L-AP4 \( \text{pEC}_{50} \) in high-Cl\(^-\) buffer and showed no variations in low-Cl\(^-\) buffer compared with the WT (Supplemental Table S2). However, both mutations resulted in a significant increase in L-AP4 \( \Delta \text{pEC}_{50} \) (1-way ANOVA with Dunnett’s posttest values of \( P < 0.001 \) and 0.01, respectively, compared with the WT), suggesting that Phe56 and Val161 may play an indirect role in Cl\(^-\) sensitivity. The S110A mutation increased the concentration-response curve maximum in both buffers and also increased L-AP4 potency in the low-Cl\(^-\) buffer but did not seem to be involved in Cl\(^-\) sensitivity.

Concerning the site-directed mutagenesis of Cl\(^-\) binding site 2, S159A and Y230A mutations decreased L-AP4 potency in high- and low-Cl\(^-\) buffers. But these mutations did not affect Cl\(^-\) sensitivity and belong to the amino acid signature motif involved in orthosteric agonist binding. Moreover, neither S229A nor N286A mutants showed significant changes in \( \Delta \text{pEC}_{50} \). S160A mutant also did not...
show effects on chloride sensitivity, even by replacing it with a negatively charged amino acid to mimic Cl\(^-\) binding (S160D mutant) (Fig. 5F and Supplemental Table S2). However, according to modeling data, this residue seemed to be directly involved in Cl\(^-\) binding. We speculate that the influence of chloride on site 2 mutant activity might be hidden by the drastic effect of Cl\(^-\) removal in site 1. To overcome this issue, we used double mutants carrying the T108E mutation, which mimics Cl\(^-\) binding in site 1. Interestingly, TESA (T108E plus S160A) mutant functional response was significantly reduced in both Cl\(^-\) buffers (Fig. 5D). Substituting both Thr108 and Ser160 residues by a glutamate and an aspartate respectively (TESD mutant) mimicked Cl\(^-\) binding and rescued the receptor signaling, which becomes insensitive to Cl\(^-\) depletion (E and F). Relative fluorescence units were normalized to each data set baseline and to the maximum response of the WT in high-Cl\(^-\) buffer. Expression data and concentration-response curve parameters are shown in Supplemental Fig. S1C and Supplemental Table S2, respectively. ND, not determined; VEH, effect of free-agonist buffer (vehicle) on Ca\(^{2+}\) mobilization. One-way ANOVA with Dunnett’s posttest P value: ***P < 0.001 compared with WT.

Variations of extracellular chloride significantly modulate glutamate potency on mGlu4, but not on mGlu2

In order to evaluate the role of Cl\(^-\) in modulating mGlu4 and mGlu2 signaling activity, we performed calcium mobilization assays in buffers containing different [Cl\(^-\)], ranging from 2 to 154.6 mM (Fig. 6A,B). Cl\(^-\) chemical species were replaced by gluconate equivalents to maintain the osmolarity of the medium. [Cl\(^-\)] had a dose-dependent effect on glutamate potency. The mGlu4 functional response was significantly rescued with increased [Cl\(^-\)] (Fig. 6A, left), whereas the mGlu2 concentration-response curve showed relatively small variations (Fig. 6A, right). Therefore, differences in mGlu4 glutamate pEC\(_{50}\) were significant for all Cl\(^-\) concentrations tested compared with [Cl\(^-\)] (154.6 mM; Fig. 6B, left). On the contrary, differences in mGlu2 values were relatively small and became significant for the concentrations of Cl\(^-\) <50 mM (1-way ANOVA with Dunnnett’s posttest value of P < 0.01) (Fig. 6B, right). The Ca\(^{2+}\) signaling response at a fixed concentration of glutamate (10 \(\mu\)M) was plotted against [Cl\(^-\)] (Table 1). This concentration corresponds to a concentration engendering 80% of the maximal response measured in high-Cl\(^-\) buffer (EC\(_{50}\)). Chloride EC\(_{50}\) was found to be ~60 and 80 mM for mGlu2 and mGlu4, respectively. Interestingly, the Hill

Figure 5. Molecular basis of mGlu4 chloride sensitivity. Calcium mobilization assays of rat mGlu4 WT (A), T108A (B), T108E (C), TESA (D), and TESD (E) mutants transiently expressed in HEK293 cells with a chimeric G protein to allow intracellular calcium measurements. Instead of glutamate, L-AP4 was used as agonist to allow quantification of Cl\(^-\) sensitivity by ΔpEC\(_{50}\) calculations (F). Directed mutagenesis was performed according to molecular modeling and shows the involvement of Thr108 in Cl\(^-\) binding in site 1 in the mGlu4 extracellular domain: T108A mutation abolishes functional response (B), whereas mimicking Cl\(^-\) binding by replacing the threonine by a glutamate (T108E) partially rescues the functional response (C). The receptor remains sensitive to Cl\(^-\) due to the presence of site 2. Mimicking Cl\(^-\) binding in site 1 (T108E mutation) combined to the S160A mutation (TESA) in putative Cl\(^-\) binding site 2 abolishes functional response (D). Replacing this Ser160 residue by an aspartate (TESD) mimics Cl\(^-\) binding and rescues the receptor signaling, which becomes insensitive to Cl\(^-\) depletion (E and F). Relative fluorescence units were normalized to each data set baseline and to the maximum response of the WT in high-Cl\(^-\) buffer. Expression data and concentration-response curve parameters are shown in Supplemental Fig. S1C and Supplemental Table S2, respectively. ND, not determined; VEH, effect of free-agonist buffer (vehicle) on Ca\(^{2+}\) mobilization. One-way ANOVA with Dunnett’s posttest P value: ***P < 0.001 compared with WT.
The number was 2.04 ± 1.12 for mGlu2 and increased to 5.94 ± 1.71 for mGlu4, supporting a higher cooperativity between glutamate and Cl\textsuperscript{−} ions in mGlu4 (which possesses 2 functional Cl\textsuperscript{−} binding sites in each protomer) than in mGlu2 (which possesses only 1 functional Cl\textsuperscript{−} binding site by protomer) (Fig. 6C).

In order to evaluate the individual contribution of Cl\textsuperscript{−} binding site 2 in mGlu4 Cl\textsuperscript{−} sensitivity, we performed Ca\textsuperscript{2+} mobilization assays in different [Cl\textsuperscript{−}]\textsubscript{o} for the T108E mutant, where Cl\textsuperscript{−} in site 1 is mimicked by the carboxylate moiety of a glutamate residue. The T108E functional response to glutamate was significantly rescued by increased [Cl\textsuperscript{−}]\textsubscript{o}, similarly to mGlu4 WT (Supplemental Fig. S2A). Differences in pEC\textsubscript{50} were significant for 25, 50, and 90 mM compared with [Cl\textsuperscript{−}]\textsubscript{o} (154.6 mM; Supplemental Fig. S2B).

Interestingly, mGlu4 WT and T108E Cl\textsuperscript{−} concentration-response curves at glutamate EC\textsubscript{50} were similar (Supplemental Fig. S2C, left), and the chloride EC\textsubscript{50} was ~80 mM for both receptors (Supplemental Fig. S2C, right), suggesting that mGlu4 Cl\textsuperscript{−} modulation may predominantly occur in site 2.

### Extracellular chloride changes in the synaptic cleft during GABAergic synaptic events may be compatible with a modulation of mGlu4 signaling

We subsequently assessed if Cl\textsuperscript{−} dependence on mGlu2 and mGlu4 signaling was compatible with changes in [Cl\textsuperscript{−}]\textsubscript{o} occurring during a synaptic event. There is a lack of direct physiologic evidence in the literature describing variations of [Cl\textsuperscript{−}]\textsubscript{o}. This is largely due to the fact that extracellular Cl\textsuperscript{−} probes are not yet available, and the integrated role of Cl\textsuperscript{−} makes it hard to perform electrophysiologic experiments on native tissues in medium depleted in Cl\textsuperscript{−}.

Given the difficulty of experimentally assessing rapid and localized fluctuations in [Cl\textsuperscript{−}]\textsubscript{o}, we resorted to computational modeling to assess the amplitude of activity-related changes in [Cl\textsuperscript{−}]\textsubscript{o} in the vicinity of GABA\textsubscript{A} synapses, where presynaptic group III mGluRs, notably mGlu4 receptors, are located at the synaptic grid and modulate GABAergic transmission carried by Cl\textsuperscript{−} flux through GABA\textsubscript{A} receptors (7, 41–43).

We modeled electrodiffusion in the extracellular space according to Poisson-Nernst-Planck equations (24) and based the geometry of the synaptic surroundings on the robust observation that free extracellular space consists of a connected network of thin layers running between neurons and glial cells (26, 27) (Fig. 7A, B). Our simulations...

### Table 1. Chloride potency and cooperativity on mGlu4 and mGlu2

<table>
<thead>
<tr>
<th>Receptor</th>
<th>pEC\textsubscript{50} (EC\textsubscript{50} mM)</th>
<th>Hill slope</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>mGlu2</td>
<td>1.22 ± 0.13 (59.78)</td>
<td>2.04 ± 1.12</td>
<td>4</td>
</tr>
<tr>
<td>mGlu4</td>
<td>1.11 ± 0.02 (78.60)</td>
<td>5.94 ± 1.71</td>
<td>4</td>
</tr>
</tbody>
</table>

Chloride EC\textsubscript{50} is ~60 mM for mGlu2 and 80 mM for mGlu4. The Hill slope shows a higher cooperativity of glutamate with chloride ions in mGlu4 than in mGlu2. Data ± sst are represented.
indicate that a single hyperpolarizing GABA$_A$ event can cause instantaneous decrease in [Cl$^-$]$_o$ of the order of 20 mM in the synaptic cleft (Fig. 7C, D). Chloride flux through GABA$_A$ synapses can either be inward or outward depending on various conditions such as the [Cl$^-$]$_i$ level concentration and the membrane potential (44). Our simulations also indicate that depolarizing GABA$_A$ activity increases [Cl$^-$]$_o$ by $\sim 20$ mM in the synaptic cleft (Fig. 7E, F), a change of similar magnitude to the one associated with a single hyperpolarizing event, but in the opposite direction.

Furthermore, our modeling indicates that repeated synaptic activation makes the activity-dependent change in

Figure 7. Computational modeling of activity-related changes in [Cl$^-$] at GABAergic synapses. A) Schematic of the modeled geometry. The free extracellular space (in black) consists of sheets of 20 nm thickness. White arrows indicate the direction of the Cl$^-$ flow. B) Three-dimensional tetrahedral mesh used to solve the Poisson-Nernst-Planck equations; [Cl$^-$] and electric potential were computed at each node of the mesh. C) Sample color-coded image of [Cl$^-$] in the vicinity of a GABA$_A$ synapse during a single hyperpolarizing event (taken at the maximum of the GABA$_A$ current). D) Time course of GABA$_A$ synaptic conductance (top) and [Cl$^-$] in the synaptic cleft (bottom) during a single hyperpolarizing GABA$_A$ event assuming a maximal synaptic conductance of 2 nS and a membrane potential of $-40$ mV (due to concurrent excitatory and firing activity). E) Sample color-coded image of [Cl$^-$] in the vicinity of a GABA$_A$ synapse during a single depolarizing event (taken at the maximum of the GABA$_A$ current). F) Time course of GABAergic conductance (top) and of the [Cl$^-$] response at the synapse (bottom) in response to a single GABAergic event assuming a holding potential of $-65$ mV and a maximal conductance of 2 nS. G) We submitted the model to joint excitatory and inhibitory activity at 20 Hz for 200 ms. Left panel shows the time course of synaptic conductance. Right panel shows the response in terms of [Cl$^-$] in the synaptic cleft and in terms of membrane potential near the synapse.
[Cl\textsuperscript{−}], more dramatic (Fig. 7G), as is the case for intracellular Cl\textsuperscript{−} accumulation (45). Indeed, when simulating a train of GABA\textsubscript{A} synaptic events occurring at 20 Hz during 200 milliseconds as well as concurrent depolarizing activity (e.g., action potential firing or glutamatergic inputs), [Cl\textsuperscript{−}], fluctuations in the synaptic cleft reached 25 mM (Fig. 7G). The magnitude of the activity-dependent [Cl\textsuperscript{−}]\textsubscript{o} fluctuations (either due to a single event or to repeated activity) depended on the strength of the synaptic Cl\textsuperscript{−} current rather than on its direction or on the baseline [Cl\textsuperscript{−}]\textsubscript{o} value.

Considering the range of variations in [Cl\textsuperscript{−}]\textsubscript{o}, that we estimated to occur during GABA\textsubscript{A}-mediated synaptic events, they are unlikely to significantly affect glutamate potency at mGlu2 receptors (red rectangle in Fig. 6B, right). However, they may be sufficiently large in certain conditions to affect mGlu4 receptor function. Indeed, given the strong positive cooperativity between [Cl\textsuperscript{−}] and glutamate potency in this particular receptor, a decrease of 30 mM in [Cl\textsuperscript{−}]\textsubscript{o} corresponds to a significant increase in glutamate potency, whereas a 30 mM increase in [Cl\textsuperscript{−}]\textsubscript{o} yields a significant increase in potency (P < 0.05 for 90 mM from a baseline of 120 mM in the case of Cl\textsuperscript{−} influx; P < 0.01 for 154.6 mM from a baseline of 120 mM in the case of Cl\textsuperscript{−} efflux; red rectangle in Fig. 6B, left). Moreover, [Cl\textsuperscript{−}]\textsubscript{o} activity-related changes are predicted to add up to longer-term alterations under pathologic states such as anoxia or ischemia (46–48). Because anoxia-related changes in [Cl\textsuperscript{−}]\textsubscript{o} can exceed 30 mM (46), the total change of [Cl\textsuperscript{−}]\textsubscript{o} during synaptic events could exceed 55 mM in the synapse in anoxic conditions. This could potentially have an important impact on mGlu4 receptors (Table 2). Taken together, these data suggest that extracellular Cl\textsuperscript{−} modulation of mGlu4 signaling may be physiopathologically relevant.

**DISCUSSION**

Ion binding is required for the function of many neurotransmitter receptors, as exemplified by the action of Na\textsuperscript{+} on many GPCRs (49). Although regulation of mGluRs by extracellular cations is well documented, it has been proposed that glutamate binding on these receptors is Cl\textsuperscript{−} dependent. We report herein that all mGluRs are indeed sensitive to extracellular Cl\textsuperscript{−}, and we further define the molecular basis of this sensitivity. Moreover, for the most sensitive mGluRs, variations of [Cl\textsuperscript{−}]\textsubscript{o} in the synaptic cleft and its vicinity during GABAergic synaptic events may potentially result in a dynamic adaptation of their modulatory activity.

Several ions are known to regulate ligand binding on mGluRs. In particular, the modulation of mGlu1 by Ca\textsuperscript{2+} has been extensively studied (14, 50–54), and several sites for divalent cations have been predicted (11, 12, 55) based on crystal structures of the extracellular domains of mGlu1, mGlu5, and mGlu7 (13, 56, 57). The first evidence for Cl\textsuperscript{−} dependency of ligand binding was provided by Eriksen and Thomsen (16), who showed that this anion increased the binding of the radiolabeled agonist L-AP4 on mGlu4. Later, it was shown that Cl\textsuperscript{−} also improves the binding of the nonselective antagonist LY341495 on group II and group III mGluRs (17, 58). Cl\textsuperscript{−} influence on ligand binding was further confirmed by Kuang and Hampson (14) on truncated versions of the receptors only constituted of the extracellular domain of the receptor. However, the precise site of action of Cl\textsuperscript{−} was still unknown. In the present study, we confirm that all mGluRs are sensitive to Cl\textsuperscript{−}, to different extents, and that this sensitivity is carried by binding sites located in their extracellular domains.

We identified 2 functional Cl\textsuperscript{−} binding pockets in the extracellular domain of all mGluRs, except in mGlu2, which possesses only 1 functional site. The first Cl\textsuperscript{−} binding site is found in lobe 1 of the extracellular domain of the receptors. Interestingly, this site is well conserved not only in mGluRs but also other members of the LIVBP-like family. It corresponds to the Cl\textsuperscript{−} binding site of the structurally closely related natriuretic peptide receptor (NPR), NPR-A (59). In mGluRs, this site was previously described as the binding pocket for the carboxylate moiety of the mGlu4-selective agonist LSP4-2022, and the putative presence of a chloride ion was considered (15, 60). Analysis of sequence alignments of NPRs, mGluRs, and other class C GPCRs [notably the calcium-sensing receptor (CaSR), GPRC6A, the olfactory receptor 5.24, and the taste receptor T1R3 (61–63)] revealed that the Ser53 in NPR-A, which coordinates Cl\textsuperscript{−} through its polar side chain, is either conserved or replaced by a similar amino acid (threonine or asparagine) in other receptors (15, 59). In the case of mGluRs, this serine is conserved in mGlu1, mGlu2, and mGlu5 and is replaced in mGlu3 and group III mGluRs by a threonine, which, as we have demonstrated in this study, is crucial for Cl\textsuperscript{−} binding. The other residues that coordinate Cl\textsuperscript{−} are less conserved between the different receptors. The site is located between 2 highly conserved short loops that connect 2 β-sheets to 2 α-helices (β2-α2 and β3-α3), close to the dimeric interface between helices α2 and α3. This location suggests that the role of Cl\textsuperscript{−} (or another bridging moiety) would be structural, maintaining the 2 helices and stabilizing the VFT conformation, which allows dimerization and activation.

**Table 2. Summary of sources and extent of [Cl\textsuperscript{−}]\textsubscript{o} fluctuations in the synaptic cleft**

<table>
<thead>
<tr>
<th>Type of GABA\textsubscript{A} activity</th>
<th>Single GABA\textsubscript{A} event</th>
<th>Repeated GABA\textsubscript{A} events</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anoxia (mM)</td>
<td>20–30</td>
<td>20–30</td>
</tr>
<tr>
<td>GABA\textsubscript{A} event(s) (mM)</td>
<td>~20</td>
<td>~25</td>
</tr>
<tr>
<td>Total change (mM)</td>
<td>40–50</td>
<td>45–55</td>
</tr>
</tbody>
</table>

Although changes due to anoxia affect the baseline concentration, dynamic changes due to Cl\textsuperscript{−} currents should add to these baseline changes so that total fluctuations of Cl\textsuperscript{−} concentration at the mGluR location can exceed 50 mM, having an important modulatory impact on the presynaptic receptor.
The second Cl− binding site is located at the surface of lobe 2, in the cleft between the 2 lobes, close to the glutamate binding pocket. This site is conserved in all mGluRs, except in mGlu2 where site 2 is occupied by the negatively charged carboxylate moiety of Asp146, which likely mimics Cl−. This carboxylate moiety makes hydrogen bonds with the NH backbone of Tyr216 and a salt bridge with Arg271, a residue involved in interlobe connections through a cation-π interaction with Tyr144 and a salt bridge with Glu273 (64), as confirmed in a recent crystal structure of mGlu2 (65). Interestingly, mGlu2 is also the least-sensitive mGluR to Cl−. The location of site 2 at the interface between the 2 lobes suggests that the role of Cl− is to stabilize the closed active conformation of the VFT upon glutamate binding, by making contact between lobe 1 and lobe 2, thus facilitating receptor activation.

Given the dimeric nature of mGluRs (10), our data suggest that there are 2 glutamate binding sites and 4 functional Cl− binding sites per dimer (except for mGlu2). Consequently, functional positive cooperativity of Cl− and glutamate is very high on most mGluRs (Hill slope ∼6 at mGlu4 receptors for example), meaning that a small variation in [Cl−] may lead to a large variation in glutamate action. Cl− potency on glutamate activity (∼80 mM on mGlu4) is close to the estimated resting value of [Cl−]o (110–120 mM). Taken together, the Cl− dependence on glutamate activity, Cl− potency, and the localization and conservation of the Cl− binding sites strongly suggests a regulatory role for bound Cl−.

The Hill coefficient determined from the [Cl−]-response curve obtained from calcium mobilization data at glutamate EC50 is theoretically superior to the number of Cl− binding sites described in this study. Because the assay used was a functional assay and does not directly reflect Cl− binding, we cannot exclude a flaw in Hill slope estimation. However, FRET measurement data in increased Cl− concentration have shown similar values of Hill slope (for mGlu4, pH = 5.94 ± 1.71 in Ca2+ assay and 6.20 ± 1.59 in sensor assay). High cooperativity values were also described for another class C GPCR, the CaSR, in which 5 Ca2+ binding sites per protomer have been predicted (66, 67). Thus, one possibility is that there are >2 Cl− binding sites per protomer. Recent crystal structures of mGlu2 and mGlu5 extracellular domains showed other putative Cl− binding sites, in addition to those described in this study (65). Some of these additional Cl− sites are closely located to the position of Ca2+ binding sites in CaSR. Interestingly, CaSR senses both Ca2+ and l-amino acids, such as l-Phe. In that case, a regulating Ca2+ binding site communicates with the l-amino acid binding pocket (corresponding to glutamate binding pocket in mGluRs) and mediates positive cooperativity between Ca2+ ions and the amino acid during activation (67). Moreover, in this receptor, concept of functional positive homotropic cooperativity was named as molecular connectivity between all Ca2+ binding sites, centered to 1 Ca2+ site located in the cleft, responsible for the heterotropic cooperativity with l-amino acids and crucial for receptor activation. This site corresponds to Cl− binding site 2 in mGlu4. Such a mechanism might be possible in mGluRs carrying multiple Cl− binding sites, and as a consequence, our mutagenesis data highlighted “regulating” Cl− binding sites displaying the most functional significance. Indeed, CaSR mutants, especially those containing mutations close to the hinge region between lobe 1 and lobe 2, cause a disruption of the heterotropic cooperativity between the Ca2+ binding site and l-Phe (67). Another possibility to explain high cooperativity values between Cl− and glutamate at mGlu4 is to consider the oligomeric state of mGluRs. However, previous studies have shown that mGluRs form strict dimers (homodimers and heterodimers) in contrast to the GABAergic receptor (39, 68). Interestingly, mGlu2 oligomers can transiently form if in their active state. Although the latter were too transient to detect FRET between dimers, their close contact could be trapped by cysteine cross-linking (38). Further studies are needed to explore these hypotheses and assess more precisely cooperativity phenomena in mGluRs.

Targeting the Cl− binding sites may be of interest for the development of mGluR ligands. For example, the first selective and potent orthosteric agonist of mGlu4, LSP4-2022, is an extended derivative of glutamate with a glutamate-like moiety binding in the orthosteric binding sites and a carboxylate distal moiety binding into the Cl− binding site 1 (60). Selectivity for mGlu4 is due to topological differences in the Cl− site 1. In the future, it would also be interesting to develop a new generation of allosteric regulators targeting Cl− binding sites. This may lead to allosteric modulators exhibiting a very strong cooperativity with glutamate, higher, by far, than the best allosteric modulators currently available for mGluRs and that are considered as valuable therapeutic tools.

[Cl−] varies at various time scales ranging from a few milliseconds to several days in response to diverse factors, such as alterations in transport activity, synaptic activity, and constituents of the extracellular matrix. Long-term changes can occur as consequences of pathology (69, 70) and maturation (44). For instance, [Cl−], decreases from >30 mM in the immature to a 5–10 mM range in the adult (33), whereas down-regulation of the Cl− exporter KCC2 in various pathologic states leads to an increase of [Cl−], of >10 mM (1). [Cl−]o has been shown to vary greatly as a result of pathogens such as ischemia, anoxia, and changes in the content of the extracellular matrix (46–48). For example, anoxia-related changes in [Cl−]o concentration can exceed 30 mM (46). On the other hand, fast changes of [Cl−]o resulting from rapid influx or efflux of Cl− through GABA or glycine-gated ion channels are occurring in the 10 ms to 1 s time scale due to neural activity (71). The extent of these short-term activity-related [Cl−] fluctuations is determined by the capacity of Cl− ions to diffuse between different cell compartments and the size of the cell compartment in which the activity occurs. For example, the thin diameter of a distal dendrite will slow Cl− diffusion toward the soma, exacerbating local accumulation (72). Such dynamic fluctuations should also occur in the extracellular space because its convoluted geometry limits diffusion and compartmentalizes ionic concentrations (29, 30), especially near synapses that are often wrapped in a dense network of extracellular matrix (73).

Group II and III mGluRs are mostly presynaptic receptors, and their activation tends to reduce synaptic transmission and neuronal excitability. As autoreceptors, they are involved in reducing transmission at glutamatergic synapses, but they are also heteroreceptors found in GABAergic terminals where they reduce GABA release
at inhibitory synapses (41–43). One can wonder whether fast and slow changes in [Cl\(^{-}\)] occurring at GABAergic synapses may result in a variation of mGlu heteroreceptor ability to modulate transmission. Due to the lack of extracellular Cl\(^{-}\) probes and the difficulty to perform electrophysiologic studies in native tissues in medium depleted in Cl\(^{-}\), there is a lack of direct physiologic evidence in the literature describing variations of extracellular Cl\(^{-}\) concentrations. As an alternative, we modeled the fast variations in [Cl\(^{-}\)]\(_o\) that can occur in the vicinity of GABAA synapses during a synaptic activity. In this electrodiffusion model, we found that the extent of [Cl\(^{-}\)]\(_o\) is dependent on synaptic driving force, synaptic conductance, synaptic activity, and geometry but independent of whether the Cl\(^{-}\) current was hyperpolarizing or depolarizing. We observed changes in [Cl\(^{-}\)]\(_o\) in the synaptic cleft that can exceed 25 mM during temporal summation of synaptic activity. Furthermore, as mentioned above, various pathologies of the CNS are associated with an altered Cl\(^{-}\) homeostasis (69) and lead to long-term changes in Cl\(^{-}\) concentration. Given that the magnitude of the activity-dependent changes in [Cl\(^{-}\)]\(_o\) was mostly independent of small changes in initial conditions, our results suggest that the fast and slow changes in [Cl\(^{-}\)]\(_o\) due to GABAergic activity and perturbation of the extracellular matrix or anoxia (46, 47) should be additive leading to variations of [Cl\(^{-}\)]\(_o\) that can reach up to 45–55 mM for multiple events. These variations may be compatible with a modulation of glutamate binding in the most Cl\(^{-}\)-sensitive mGluRs, especially mGlu4. Therefore, we speculate that Cl\(^{-}\)-dependent control of glutamate binding may occur during GABAA-mediated synaptic events in certain conditions and could lead to a rapid adaptation of the modulatory role of mGluRs present on GABAergic terminals. However, further studies are required to verify the physiopathologic validity of this hypothesis.

In conclusion, we demonstrate herein that chloride ions behave like PAMs of mGluRs. All mGluRs are sensitive to chloride ions compared with other GPCRs, mGlu4 being the most sensitive to extracellular Cl\(^{-}\) depletion and mGlu2 the least. The Cl\(^{-}\) sensitivity is carried by 2 sites localized in the extracellular domain of the receptors, with the remarkable exception of mGlu2 that possesses only 1 functional site. We speculate that Cl\(^{-}\) sensitivity of mGluRs could be important for the regulatory properties of those receptors present at GABAA synapses (group III mGluRs). In the future, it will be interesting to study the consequences of Cl\(^{-}\) sensitivity on synaptic modulation by mGluRs.

The authors thank Ebba L. Lagerqvist and Thierry Durroux from the Institut de Génomique Fonctionnelle for helpful discussions and critical reading of the manuscript. This work was supported by grants from the Fondation Recherche Médicale (team DEQ2013032622), the Fundación La Marató de TV3 (reference 110232), Eranet Neuron, the Agence Nationale de la Recherche (ANR-12-NEUR-0005 and ANR-13-BSV1-006), the Natural Sciences and Engineering Council of Canada (to Y.D.K.), and the Fonds de Recherche Québec-Nature et Technologie (to N.D.). Cell-based pharmacologic assays were performed on the ARPEGE (Pharmacology Screening-Interactome) platform facility at the Institut de Génomique Fonctionnelle. The Institut de Génomique Fonctionnelle belongs to the Laboratories of Excellence, Ion Channel Science and Therapeutics, and EpiGenMed. A.S.T. was supported by a Ph.D. fellowship from the Ministère de l’Éducation Nationale et de la Recherche and by the Fondation Recherche Médicale (FDT20140931071) and X.R. by the Beatriu de Pinós program of Agència de Gestió d’Ajuts Universitaris i de Recerca. I.B., J.-P.P., F.A., and C.G. were supported by the Centre National de la Recherche Scientifique. I.D. was supported by a fellowship from the Canadian Institutes of Health Neurophysics training program. A.S.T., X.R., N.D., F.A., and C.G. designed the research. A.S.T., X.R., I.D., H.-O.B., I.B., and N.D. performed the research. F.A., H.-O.B., and N.D. contributed new reagents or analytic tools. A.S.T., X.R., I.D., H.-O.B., I.B., Y.D.K., N.D., J.-P.P., F.A., and C.G. analyzed the data. A.S.T., N.D., J.-P.P., F.A., and C.G. wrote the paper. The authors declare no conflicts of interest.

REFERENCES


for developing subtype selective ligands with therapeutic potential. FASEB J. 26, 1682–1693


Allosteric modulation of metabotropic glutamate receptors by chloride ions

Amélie S. Tora, Xavier Rovira, Ibrahima Dione, et al.

FASEB J published online June 26, 2015
Access the most recent version at doi:10.1096/fj.14-269746
Supplemental figure 1: Mapping chloride binding sites in mGlu4 and mGlu2 extracellular domains.

A) Molecular modeling of mGlu4 VFT based on the crystal structure of mGlu1 (PDB 1EWK) and mGlu3 VFTs (PDB 2E4U and 3SM9), displaying two chloride binding sites. Putative amino acids involved in chloride binding in site 1 (orange) are located in lobe 1 while residues surrounding site 2 (blue) belong to the interface of the two lobes. B) Multiple sequence alignment of the eight mGluRs. Amino acids putatively involved in chloride binding are orange (site 1) or blue (site 2). Purple color intensity is correlated with the percentage of amino acid conservation. Numbers correspond to mGlu4 amino acid numbering. C) Expression of mGlu4 and mGlu2 mutants. HEK293 cells were transiently transfected with N-terminus HA SNAP tag mGlu2 and mGlu4 constructs and expression was quantified by ELISA assay using the primary anti-HA tag antibody coupled to peroxidase (3F10, Roche). Total expression was measured on permeabilized cells and cell surface expression on untreated cells. The low expression of G158A, V161A and N286A mutations do not affect the receptor functional activity (cf. tableS2). Group data of at least three experiments.
Supplemental figure 2: Chloride modulation of mGlu4 T108E.

(A) Calcium mobilization assay in HEK293 cells transiently expressing rat mGlu4 T108E. Glutamate concentration-responses curves were obtained in buffers with chloride concentration ranging from 2 mM to 154.6 mM. Glutamate potency (pEC_{50}) shift was analyzed in (B). Red rectangle correspond to chloride concentrations estimated by synaptic event modeling (see fig. 7).

Group data of at least three experiments. Data are normalized by the maximum response (top of the curve) obtained in 154.6 mM chloride buffer in each experiment and are analyzed with a four-parameter concentration-response curve equation. pEC_{50} values represent the mean ± S.E.M. and statistical significance was analyzed using one way ANOVA test with a Dunnett's post-test p-value: * p<0.05, *** p<0.001, in comparison to [Cl]_o = 154.6 mM. ND: Not Determined.

(C) Chloride concentration response curve at fixed glutamate concentration (EC_{50}) (left). Calcium mobilization data obtained after stimulation with a single dose of glutamate in increased chloride concentration buffers. Dose of glutamate corresponds to a concentration engendering 80% of the maximal response measured in 154.6 mM chloride buffer (EC_{50}) for each construct of mGlu4: WT (circles) and T108E (squares). Chloride EC_{50} is around 80 mM for both mGlu4 WT and T108E (right). Data ± S.E.M. are represented.