Mechanism of Cl\textsuperscript{−} Selection by a Glutamate-gated Chloride (GluCl) Receptor Revealed through Mutations in the Selectivity Filter\textsuperscript{a}

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To learn about the mechanism of ion charge selectivity by invertebrate glutamate-gated chloride (GluCl) channels, we swapped segments between the GluClβ receptor of \textit{Caenorhabditis elegans} and the vertebrate cationic GluCl\textsuperscript{α}–acetylcholine receptor and monitored anionic/cationic permeability ratios. Complete conversion of the ion charge selectivity in a set of receptor microchimeras indicates that the selectivity filter of the GluClβ receptor is created by a sequence connecting the first with the second transmembrane segments. A single substitution of a negatively charged residue within this sequence converted the selectivity of the GluClβ receptor’s pore from anionic to cationic. Unexpectedly, elimination of the charge of each basic residue of the selectivity filter, one at a time or concomitantly, moderately reduced the \(P_{CI}/P_{Na}\) ratios, but the GluClβ receptor’s mutants retained high capacity to select Cl\textsuperscript{−} over Na\textsuperscript{+}. These results indicate that, unlike the proposed case of anionic Gly- and γ-amino Butyric acid-gated ion channels, positively charged residues do not play the key role in the selection of ionic charge by the GluClβ receptor. Taken together with measurements of the effective open pore diameter and with structural modeling, the study presented here collectively indicates that in the most constricted part of the open GluClβ receptor’s channel, Cl\textsuperscript{−} interacts with backbone amides, where it undergoes partial dehydration necessary for traversing the pore.

The invertebrate GluCl\textsuperscript{β} receptor channels are pentameric transmembrane receptors belonging to a wide superfamily of Cys-loop receptors activated by various neurotransmitters such as acetylcholine (ACh), serotonin (5-hydroxytryptamine, 5HT), γ-amino Butyric acid (GABA), Gly, Glu, or histamine (Fig. 1A) (1–8). This superfamily consists of cationic channels permeable to Na\textsuperscript{+}, K\textsuperscript{+}, and, in many subunit combinations, to Ca\textsuperscript{2+} ions, as well as of anionic channels selective to Cl\textsuperscript{−} ions (reviewed by Keramidas et al. (9)). Structural similarities shared by Cys-loop receptors enabled swapping of pore sequences between cationic and anionic channels so as to assess the involvement of specific amino acids in ion charge selectivity. It was previously shown that concomitant replacement of the residues at positions −2', −1', and 13' (Fig. 1, B and C) of cationic receptors by the residues found at the homologous positions of anionic receptors, and vice versa, lead to conversion of ion charge selectivity (10–13).

Further mutagenesis studies led to the recognition that the different capacities of cationic versus anionic Cys-loop receptors to distinguish between the charge of ions rely on the differences in the amino acid composition at positions −1 and −2 (Fig. 1C) (13–16). The conserved pore-facing Glu residue at position −1' of cationic Cys-loop receptors was further inferred to form, around the axis of ion conduction, a negatively charged ring that plays the key role in cationic selectivity by interacting with cations and repulsing anions (12, 13, 15, 16). Conversely, a conserved arginine at position 0' of anionic Cys-loop receptors was inferred to interact with anions and repulse cations. A basic residue at position 0' is also typical of all cationic Cys-loop receptors (Fig. 1C and the ligand-gated ion channels data base), but it was suggested that local conformational differences in the M1-M2 connecting segment (M1-M2 loop) orient this basic residue to the pore lumen only in anionic Cys-loop receptors (9, 15). These local conformational differences have been attributed to a proline residue, which is present exclusively at position −2' of anionic Cys-loop receptors (12, 14–17).

Unlike all other homomeric anionic Cys-loop receptors studied thus far, the β subunit of the GluCl receptor does not have a proline residue at position −2', a feature that minimizes the likelihood of causing drastic local conformational changes when mutating its M1-M2 loop. As the GluClβ subunit can assemble into a functional anionic homomeric receptor (GluClβR) (18), the selectivity filter of the GluClβR was readily identified here by microchimerism and then was extensively mutated. Electrophysiological analyses of anionic permeability ratios in a large repertoire of mutants, together with computer-assisted molecular modeling, reveal a novel mechanism of Cl\textsuperscript{−} selection by a Cys-loop receptor.

**EXPERIMENTAL PROCEDURES**

**Chimeras and Mutants**—The α7-GluClβ chimeric subunit was prepared as performed previously with the α7-5HT\textsubscript{1A}R (19), by fusing the N-terminal half of the chick α7 subunit (Fig. 1A, red segment) (Swiss-Prot accession number P22770) to the C-terminal half of the β subunit of the GluClR (Fig. 1A, non-red segments) (Swiss-Prot accession number Q17328). The entire sequence of the chimera is provided in Supplemental Fig. S1. Mutations were introduced as described previously (20).

**Electrophysiology**—Human embryonic kidney (HEK-293) cells were transfected with the various chimeras together with a green fluorescent...
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Chimeric Design and Current Amplitudes of the Various Mutants—As a first step, we generated a chimeric subunit where the extracellular segment of the AChR α7 subunit was fused to the GluClβ subunit segment that folds in the membrane and cytoplasm (see “Experimental Procedures” and supplemental Fig. S1). The α7-GluClβ chimera subunit assembles into a homopentameric receptor (α7-GluClβR), like the

mined at 25 °C by two methods. (i) Currents evoked by 100 μM ACh for 3 s were measured at different holding potentials ranging from −100 to +50 mV, and (ii) inverted 200-ms long voltage ramps (either from +50 to −100 mV or from +70 to −100 mV) were applied 1 and 2 s after the beginning of a 3-s ACh application. The initial holding potential was −60 mV. Leak currents obtained by the same protocol, in the absence of ACh, were subtracted. Measured reversal potential ($E_{\text{rev}}$) values were corrected to account for the liquid junction potentials by using the JPCalc software (21) implemented in pClamp version 8.1. Permeability ratios for Na⁺, Cs⁺, and Cl⁻ were calculated using the Goldman-Hodgkin-Katz equation,

$$E_{\text{rev}} = \frac{RT}{F} \ln \left( \frac{[\text{Cl}^-]_{\text{in}} + \alpha [\text{Na}^+]_{\text{out}} + \beta [\text{Cs}^+]_{\text{out}}}{[\text{Cl}^-]_{\text{out}} + \alpha [\text{Na}^+]_{\text{in}} + \beta [\text{Cs}^+]_{\text{in}}} \right)$$

where $R$, $T$, and $F$ are the gas constant, the absolute temperature, and the Faraday’s constant, respectively, and the permeability ratios are $\alpha = P_{\text{Na}}/P_{\text{Cs}}$ and $\beta = P_{\text{Cs}}/P_{\text{Cl}}$. Note that ion activities, instead of ion concentrations, have been used in the analyses of permeability ratios. Ionic activities were calculated on the basis of the Debye-Hückel theory (22) but with the corrections introduced in the Millero-Pitzer method for solutions having an ionic strength greater than 0.1 M (41), as implemented in the Electrolytes program of Aq-Solutions, a software package of programs for the quantitative treatment of equilibria in solution.

Model Building—An initial homology model was built by using the atomic coordinates of the AChR structure (23) as a template (Protein Data Bank number 1OED), as recently described (20). After modeling the M1-M2 loop, it was tilted together with the M1 and M2 segments to an intermediate position between the closed and open states previously elaborated by Paas et al. (20) in the case of another Cys-loop chimeric receptor. We readily obtained an intermediate position displaying a distance of ∼6.1 Å between the van der Waals surfaces of backbone amides located on opposite sides of the pore, at the level of position −3. This distance is within the range of the effective open pore diameter determined here. Note that the M1-M2 loop of the GluClβR (DLH- STAG) is shorter by two amino acids than the loop of the models elaborated in Paas et al. (20) (PPDLHSTAG). As a result, when compared with its position in the chimera modeled in Paas et al. (20), histidine(H)−5’ of the GluClβR’s pore (modeled here) moved away from the permeation pathway. Consequently, H−5’ of the GluClβR’s model is not in contact with the permeating ions. This modeling observation is in line with the incapacity of Zn²⁺ to block the chimeric α7-GluClβR (data not shown), unlike the case of a chimera having the transmembrane segments of the 5HT₃A to the sequence PPDLHSTAG between M1 and M2 (20). The model of chimera 17 was built as above but with a proline, instead of alanine, at position −2’. The root mean square difference between the backbone atoms of the GluClβR and chimera 17 is 0.03 Å (throughout the pentameric membrane-embedded domains). The root mean square difference between the backbone atoms of the M1-M2 loops (plus their flanking residues, 1−10’ and 20’) of these structures is 0.08 Å.

RESULTS

For further information about this software package, please contact the author (Y. P.).
Ion Charge Selectivity in an Anionic Cys-loop Receptor

The I-V relations plotted for the α7-GluClβR show that omission of almost all external Cl⁻ ions shifts the reversal potential (E_{rev}) to a positive voltage (e.g. Fig. 2B), by 54.5 ± 1.2 mV (mean ± S.E. from 12 cells, after correcting with liquid junction potentials). The latter value is close to the maximal theoretical shift calculated based on the Cl⁻ equilibrium (Nernst) potential (61.4 mV; calculated using ionic activities). The extent of the shift depended on the external concentrations of Cl⁻ (Fig. 3A) and showed that the α7-GluClβR chimera is highly selective to Cl⁻ relative to Na⁺ and Cs⁺ (P_{Cl}/P_{Na} = 45 and P_{Cl}/P_{Cs} = 26; Table 1), as expected from a chimera harboring the pore of the GluClβR (18). Previous studies suggested that a positively charged residue substituted at the extracellular mouth of Cys-loop receptor mutants (position 19') partially contributes to ionic selectivity by attracting Cl⁻ ions (24) or repulsing divalent cations (15). It was also shown that an Arg^{19'} → Glu mutation in an anionic-to-cationic converted glycine receptor mutant contributes to cationic conductance (25). We therefore replaced the

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**Table 1**

Permeability ratios (P_{Cl}/P_{Na}) determined for the chimeric α7-GluClβR and its mutants

Note that: (i) data are means ± S.E.; (ii) all values were rounded to the closest decimal number; (iii) pair sequence alignment between the α7 and the GluClβR receptors dictates the movement of the α7-G'→3' alignment in Fig. 1C by one position downstream; (iv) gaps in the sequence are also numbered; (v) residues shown in blue do not appear in the native sequence of either the α7-ACHR or the GluClβR; (vi) the entire sequence of the α7-GluClβR chimeric subunit is provided in the Supplemental Data (Fig. S1).

| M1 | 7- | 1- | 2' | 19' | I (nA)^a | P_{Cl}/P_{Na} | P_{Cl}/P_{Cs} | N^b | S^c |
|----|----|----|----|-----|--------|-------------|-------------|-----|-----|
| α7-ACHR: | FLLPAD | S | GKEISLGTVTLTVFMLLVMEIMP | 3.8 ± 0.7 | 45.2 ± 4.9 | 26.0 ± 1.9 | 12 | A |
| GluClβR: | WNI | - | DPGVAGVTITLTMQSAIKNLP | 3.1 ± 0.4 | 18.4 ± 1.2 | 28.8 ± 2.0 | 6 | A |
| Chimera 1: | WNI | - | DPGVAGVTITLTMQSAIKNLP | 0.8 ± 0.05 | 17.3 ± 1.4 | 19.4 ± 1.6 | 8 | A |
| Chimera 3: | WNI | - | DSGKVLTVTLLTMQSAIKNLP | 1.0 ± 0.3 | 0.02 ± 0.002 | 0.02 ± 0.004 | 5 | C |
| Chimera 4: | WNI | - | DSGKVLTVTLLTMQSAIKNLP | 3.4 ± 1.0 | 0.02 ± 0.002 | 0.01 ± 0.003 | 7 | C |
| Chimera 5: | WNI | - | DSGKVLTVTLLTMQSAIKNLP | 1.9 ± 0.5 | 0.03 ± 0.01 | 0.01 ± 0.004 | 4 | C |
| Chimera 6: | WNI | - | DSGKVLTVTLLTMQSAIKNLP | 2.9 ± 0.7 | 0.07 ± 0.004 | 0.05 ± 0.006 | 6 | C |
| Chimera 7: | WNI | - | DSGKVLTVTLLTMQSAIKNLP | 3.2 ± 0.9 | 0.10 ± 0.008 | 0.08 ± 0.007 | 7 | C |
| Chimera 8: | WNI | - | DSGKVLTVTLLTMQSAIKNLP | 4.4 ± 1.0 | 0.03 ± 0.01 | 0.03 ± 0.007 | 5 | C |
| Chimera 9: | WNI | - | DSGKVLTVTLLTMQSAIKNLP | 2.6 ± 0.3 | 0.08 ± 0.003 | 0.06 ± 0.003 | 7 | C |
| Chimera 10: | WNI | - | DSGKVLTVTLLTMQSAIKNLP | 1.5 ± 0.3 | 53.1 ± 6.14 | 15.3 ± 0.92 | 6 | A |
| Chimera 11: | WNI | - | DSGKVLTVTLLTMQSAIKNLP | 1.5 ± 0.3 | 24.8 ± 1.7 | 21.0 ± 2.0 | 6 | A |
| Chimera 12: | WNI | - | DSGKVLTVTLLTMQSAIKNLP | 4.2 ± 1.1 | 24.4 ± 2.8 | 20.7 ± 2.3 | 7 | A |
| Chimera 13: | WNI | - | DSGKVLTVTLLTMQSAIKNLP | 3.5 ± 0.9 | 52.7 ± 4.3 | 10.3 ± 0.7 | 6 | A |
| Chimera 14: | WNI | - | DSGKVLTVTLLTMQSAIKNLP | 5.3 ± 0.7 | 21.7 ± 1.4 | 11.6 ± 0.95 | 11 | A |
| Chimera 15: | WNI | - | DSGKVLTVTLLTMQSAIKNLP | 0.8 ± 0.1 | 24.3 ± 1.5 | 7.58 ± 0.80 | 6 | A |
| Chimera 16: | WNI | - | DSGKVLTVTLLTMQSAIKNLP | NF^f | 42.8 ± 2.3 | 21.9 ± 1.2 | 10 | A |
| Chimera 17: | WNI | - | DSGKVLTVTLLTMQSAIKNLP | 4.7 ± 0.8 | 42.8 ± 2.3 | 21.9 ± 1.2 | 10 | A |

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| a | Measured in response to 100 μM acetylcholine at -60 mV.|
|----|----|
| b | Number of cells.|
| c | Ion charge selectivity; A, anionic; C, cationic.|
| d | The P_{Cl}/P_{Na} ratio of chimeras 2 or 12 does not statistically differ from that of the α7-GluClβR (P = 0.059 and 0.150, respectively; two-tailed, unpaired t test).|
| e | The P_{Cl}/P_{Cs} ratio of chimeras 13, 14 or 15 significantly differs from that of the α7-GluClβR (P = 0.0015, 0.0025, and 0.0009, respectively; two-tailed, unpaired t test).|
| f | NF, not functional. |
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FIGURE 3. Reversal potential ($E_{rev}$) values plotted as a function of external ion activities. A, curves plotted for a few representative anionic chimeras are shown with the following open symbols: black triangles, a7-GluCl$\beta$R; inverted blue triangles, chimera 2; orange squares, chimera 11; green circles, chimera 13; red diamonds, chimera 15. The dashed line corresponds to the hypothetical situation, where $P_{Na} = 0$ and $P_{Cl} = 0.8$, curves plotted for a few representative cationic chimeras are shown with the following open symbols: black circles, chimera 4; red circles, chimera 6; green triangles, chimera 8; blue squares, chimera 9. The dashed line corresponds to the hypothetical situation, where $P_{Na} = 0$ and $P_{Cl}=2$ (calculated for chimera 4). Data points were fitted with a non-linear regression to the Goldman-Hodgkin-Katz equation implemented in pCLAMP (Axon Instruments, Inc.) (Experimental Procedures). Error bars correspond to S.E. values. The number of experimented cells is indicated in Table 1 under column N. Note that in all cases (panels A and B and all the other chimeras), replacement of external NaCl was performed by mannitol solutions containing decreasing concentrations of NaCl (see “Experimental Procedures”).

segment $^{19}$NAKL$^{22}$ of the GluCl$\beta$R pore by the homologous segment of $\alpha 7$ ($^{19}$AEIM$^{22}$), and the resulting chimera retained Cl$^-$ selectivity, albeit with a lower $P_{Cl}/P_{Na}$ ratio, but with no change in the $P_{Cl}/P_{Cs}$ ratio (chimera 1, Table 1 and Supplemental Fig. S2A). Cl$^-$ selectivity was also observed when the positive charge of the first amino acid of M2 (position 0') was neutralized (chimera 2, Table 1 and Figs. 2C and 3A).

Position −1’ is located in a constriction that extends from position 2’ toward the bottom of the pore (20, 26–30). Consistently, it was previously shown that a substitution of Glu at position 2’ of the homomeric Glyra1R converted its selectivity (31). In addition, Glu substituted at positions −3’ or −4’ of the $\beta$ subunit of a heteromeric GABA$_{A_{\alpha \beta \gamma \delta}}$ receptor impaired permeability to cations along with anions (32). Here, concomitant replacement of the M1-M2 loop and the residues at positions 0’ and 2’ of the GluCl$\beta$R’s pore by those of $\alpha 7$ resulted in a fully cationic channel (chimera 3, Table 1 and Supplemental Fig. S2B). Replacing only the M1-M2 loop also produced a fully cationic channel, indicating that a hydroxyl group at position 2’ does not play a role in ion charge selectivity (chimera 4, Table 1 and Figs. 2E and 3B). The latter conclusion well agrees with the observations that following non-polar substitutions at position 2’, the muscle AChR retains permeability to monovalent cations that are considered to interact with this position while traversing the pore (28). Cationic selectivity was also observed when chimera 4 was further modified so as to either carry the M1-M2 sequence of the cationic 5HT$_3$A$R$ (chimera 5, Table 1 and supplemental Fig. S2C) or to carry a neutral residue at position −7’ (chimera 6, Table 1, Fig. 3B, and supplemental Fig. S2D). The latter modification indicates that the so-called cytoplasmic ring (D−7’) does not play a role in ion charge selectivity. As long as a Glu residue occupied position −1’, further gradual changes in the sequence of the M1-M2 loop toward the sequence of the GluCl$\beta$R (chimera 7 and 8) did not convert the mutants back to anionic receptors, but they retained cationic selectivity, even when the GluCl$\beta$R’s pore was carrying a single G−1’E substitution (chimera 9) (Table 1, Figs. 2F and 3B, and supplemental Fig. S2E and S2F). It should, however, be noted that in three cases, a slight decrease in the relative permeability to Na$^+$ (i.e. increase in $P_{Cl}/P_{Na}$) was observed (chimeras 6, 7, and 9, Table 1).

In contrast, integrating the residues of the M1-M2 loop of $\alpha 7$ within the sequence of the GluCl$\beta$R’s loop while keeping a Gly at position −1’ (i.e. the native amino acid of the GluCl$\beta$R) provided an anionic channel (chimera 10, Table 1 and Supplemental Fig. S2G). Deleting the GluCl$\beta$R residues at positions −6’ and −5’ (LH) slightly reduced the $P_{Cl}/P_{Na}$ and $P_{Cl}/P_{Cs}$ ratios, but these mutants still displayed considerable capacity to select Cl$^-$ over Na$^+$ and Cs$^+$ (chimeras 11 and 12, Table 1, Fig. 3A, and supplemental Fig. S2H and S2I). Further deletion of the Thr that precedes position −2’ in the GluCl$\beta$R, alone (chimera 13) or together with neutralization of the charge either at position −7’ (chimera 14) or at position 0’ (chimera 15), resulted in channels that retained high Cl$^-$ over Na$^+$ selectivity but became slightly permeable to Cs$^+$ (Table 1, Figs. 2D and 3A, and supplemental Fig. S2J and S2K). Shortening the M1-M2 loop to four amino acids rendered the chimeric receptor non-functional (chimera 16, Table 1). Proline at position −2’ was shown to play a role in determining the (small) pore diameter of a glycine receptor, indicating that pore size also contributes to ion charge selectivity (Ref. 17, reviewed by Keramidas et al. (9)). Interestingly, an a7-GluCl$\beta$R having a proline at position −2’ (chimera 17) displays $P_{Cl}/P_{Na}$ and $P_{Cl}/P_{Cs}$ ratios closely similar to those of the a7-GluCl$\beta$R (Table 1 and supplemental Fig. S3).

Relative Permeability of Chloride-selective Chimeras to Isetionate and Acetate—To assess the dimensions of the open pore of the a7-GluCl$\beta$R and anionic mutants, we examined the permeability to the organic anions isetionate and acetate relatively to the permeability for Cl$^-$ . Table 2 shows that the a7-GluCl$\beta$R is not permeable to isetionate but allows acetate to permeate slightly. Elimination of the side chain at position 0’ (chimera 2) or deleting residues belonging to the M1-M2 loop of the GluCl$\beta$R (chimeras 12–15) turned these mutants permeable to isetionate and increased the relative permeability to acetate (Table 2). The increase in the relative permeability to isethionate linearly correlated with the increase in the relative permeability to acetate (Fig. 4), indicating that these mutations have effectively widened the open pore. Notably, the a7-GluCl$\beta$R A−2’P mutant (chimera 17) was found to be as permeable to acetate as the a7-GluCl$\beta$R ($P_{Acet}/P_{Cl} = 0.1 \pm 0.013$, mean ± S.E. from 5 cells e.g. supplemental Fig. S3A).

**DISCUSSION**

The Location of the Selectivity Filter in the GluCl$\beta$R’s Channel—Previous studies on the homomeric ACh–$\alpha 7$, Glyra1, and 5HT$_3$A receptors had shown that concomitant substitutions at positions −2’, −1’, and

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**TABLE 2**

Relative permeability to isethionate and acetate

| Chimera   | $P_{Cl}/P_{Na}$ | $N^*$ | $P_{Acet}/P_{Cl}$ | $N$ |
|-----------|-----------------|------|------------------|-----|
| a7-GluCl$\beta$R | 0.00 ± 0.000 | 12   | 0.09 ± 0.010     | 7   |
| Chimera 12 | 0.09 ± 0.014 | 7    | 0.22 ± 0.030     | 6   |
| Chimera 2  | 0.13 ± 0.010  | 8    | 0.25 ± 0.019     | 6   |
| Chimera 13 | 0.16 ± 0.017  | 6    | 0.37 ± 0.020     | 5   |
| Chimera 15 | 0.19 ± 0.018  | 6    | 0.46 ± 0.026     | 6   |
| Chimera 14 | 0.26 ± 0.016  | 11   | 0.56 ± 0.029     | 7   |

* Number of cells.
13’ converted their ion charge selectivity (10–14). In further experiments, an A–1’E substitution introduced concomitantly with a deletion of P–2’ in the homomeric anionic GlyIaR, GABAaR, and MOD-1 (an invertebrate Cl–-selective 5HT3AR) led to predominant cationic permeability accompanied with some permeability to Cl– (PCl/PNa = 0.13, ~0.37, and small shifts in Erev, values, respectively) (13, 15, 16). Similar changes in ion charge selectivity were observed also when an A–1’E substitution was introduced together with a deletion of A–2’ in the β subunit of an αβγδ, heteromeric GABAaR (PCl/PNa = 0.4 and PCl/PK = ~0.26) (32, 33). A single A–1’E substitution in the GlyIaR, GABAaR, and MOD-1 imparted a large component of permeability to Na+ but the mutants still allowed Cl– to permeate (PCl/PNa = 0.34, ~1.4, and small shifts in Erev, values, respectively) (13, 15, 16). Here, a single G–1’E substitution in the GluClβR’s pore (chimera 9) converted the ion charge selectivity almost completely (PCl/PNa = 0.08 and PCl/PCa = 0.06). The finding that the permeability to Cl– did not exceed 9% of the permeability to Na+ (in chimera 9) indicates that mutations that convert the ion charge selectivity unlikely induce drastic structural changes in the selectivity filter of the GluClβR’s pore. Since additional mutations toward the caticonic receptors’ sequence further reduced the PCl/PNa and PCl/PCa ratios down to 0.02 and 0.01, respectively (chimeras 8, 5, and 4), we infer as follows. In wild-type caticonic Cys-loop receptors, the M1–M2 loop shapes the selectivity filter so as to optimally orient the carboxyl moieties of position(s) −1’ in a mode that leads to (i) effective interactions with Na+ ions, (ii) repulsion of Cl– ions, and (iii) generation of a negative electrostatic potential close to the bottom of the M2 segments (34), so as to counterbalance the positive dipoles of the M2 helices.

Since replacement of the M1–M2 loop of the GluClβR by that of the α7-AChR or the 5HT3AR is sufficient to completely convert the selectivity from highly anionic to highly cationic selectivity (chimeras 4 and 5), we conclude that the apparatus acting as the selectivity filter of the GluClβR is formed by a sequence belonging to the M1–M2 loop. Based on the mild effect seen with chimera 1, we, however, do not exclude some contribution of charged residues in the outer wide vestibule to ionic permeation by affecting ionic movements and the local concentration of ions.

Positively Charged Side Chains Are Not Fundamental for Cl– Selection by the GluClβR—The capacity of caticonic Cys-loop receptors (wild types and mutants) to select cations over anions on the basis of counter charges in the selectivity filter raises the question of whether (or not) an inverse mechanism of ion charge selectivity takes place in anionic Cys-loop receptors. It was previously suggested that the residues composing the M1–M2 linker orient differently when the ion charge selectivity of the receptor is changed following mutations in this segment. It was further proposed that, unlike the case of caticonic Cys-loop receptors, the conserved basic side chain of position 0’ of the anionic Gly1aR receptor points to the pore lumen to form a positively charged ring that interacts with the passing Cl– ion and, on the other hand, repulses cations (9, 15). Here, a single R0’G mutation in the GluClβR’s pore reduced the PCl/PNa ratio only in a moderate extent, whereas the mutant retained high capability to select Cl– over Na+ and Cs+ (chimera 2). This observation indicates that positive charge at position 0’ does not play a key role in Cl– selectivity by the GluClβR. Although one may reasonably argue that the mild reduction in the PCl/PNa ratio (chimera 2) does indicate some positive-charge contribution by R0’, we assume that R0’ of the GluClβR has a role in stabilizing the shape of the selectivity filter. Computer-assisted molecular modeling indicates that the long side chain of R0’ forms multiple van der Waals interactions with H–5’, L–6’, and I–10’, on the other side of the M1–M2 loop (Fig. 5C). Elimination of this bond network might therefore destabilize the architecture of the M1–M2 loop and cause local structural changes in the main chain, also around the Ca atom of R0’.

Surprisingly, a His residue at position −5’ of the GluClβR also cannot play a key role in Cl– selectivity since chimeras 11–15, which lack this histidine, remained anionic. These observations raise the question of whether one basic side chain could compensate for the absence of the

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**FIGURE 4.** The relative permeability to isethionate plotted as a function of the relative permeability to acetate. Each data point represents an individual chimera as follows: black triangle, α7-GluClβR; inverted blue triangle, chimera 2; magenta square, chimera 12; green circle, chimera 13; gray square, chimera 14; red diamond, chimera 15. Data points were fitted with a linear regression to the equation: \( P_{\text{PCl}}/P_{\text{Cl}} = a \times P_{\text{PCl}}/P_{\text{Na}} + b \), where a is the slope, and b is the intercept with the y axis. Error bars correspond to the S.E. values provided in Table 2.

**FIGURE 5.** Structural model of the GluClβR’s selectivity filter. A, homology model of the membrane-embedded domain of the GluClβR. For clarity, only three of five differently colored subunits are shown from within the membrane. Note that the side chain of R0’ (the first amino acid of M2) points outward from the axis of ion conduction, in accord with the functional results. Carbons are shown as spheres having the color of the subunits’ ribbons, whereas nitrogen and hydrogen atoms are shown as blue and grayish spheres, respectively. B, top view of a space-filling model showing the GluClβR’s M1–M2 loop (and its flanking residues, R0’ and I–10’) organized in a 5-fold symmetry around the axis of ion conduction. Chloride is shown in magenta, with an equatorially bound water molecule. Note that the side chain of H–5’ cannot be in contact with the permeating Cl– ions (see a notion under “Experimental Procedures”). C, space-filling model that corresponds to a side view of two facing segments; each includes the GluClβR’s M1–M2 loop and its flanking residues, R0’ and I–10’. Chloride is shown in panel A. A stick model of a third subunit is shown (without hydrogen atoms) at the back. The distances between the van der Waals surfaces of two facing backbone amides at the level of A–2’ and T–3’ are 6.52 and 6.07 Å, respectively. D, a model as in panel C but of chimera 17, which has a proline at position −2. Chloride, as in panel A. The two long black arrows indicate the spheres of hydrogen atoms bonded to the C atoms of two facing prolines. The distance between the van der Waals surfaces of the closest facing hydrogen atoms of two facing prolines is 7.32 Å. The distance between the backbone amides at the level of T–3’ is 6.1 Å. In panels E–D (i) the green, blue, red, and grayish spheres correspond to carbon, nitrogen, oxygen, and hydrogen atoms, respectively; (ii) the black arrows show side chains, and the gray arrows show backbone amides; (iii) the Cl–(H2O)-water hydrogen bond used here is 2.89 Å (taken from Protein Data Bank number 1O2L), giving rise to a 6.1 Å long Cl– water complex. Note that additional water molecules can potentially be in contact with Cl–, particularly above and below the ion along the axis of ion conduction. Also note that the hydrogen bond between a Cl– ion and water can be as short as 2.46 Å (PDBe number 1C4D), so, taken together with a possible N–H–Cl hydrogen bonding, Cl– can be accommodated with an equatorially bound water molecule in anionic receptors having somewhat open porous core.
other basic residue owing to reorganization of the M1-M2 loop, which might take place when mutating the loop. In the current case, the concomitant elimination of both basic side chains (positions −5' and 0', chimera 15) did not considerably change the capacity of the receptor to select Cl− over Na+, albeit with moderately reduced $P_{Cl}/P_{Na}$ ratio and more profound decrease in the $P_{Cl}/P_{Ca}$ ratio. These observations indicate that positively charged side chains do not play a pivotal role in Cl− selectivity by the GluClBr. It further implies that the imidazole moiety of H−5' in the GluClBr's pore either is uncharged or, as predicted by the structural model (Fig. 5C), cannot be in contact with the permeating Cl− ions.

How Does the GluClBr Pore Select Cl− over Na+?—The permeability of a channel to an ion reflects the ease of an ion to enter and pass through the selectivity filter. Permeability ratios provide an estimate of the difference between the hydration energy in water and the solvation energy provided by the selectivity filter. If direct interactions of Cl− with the selectivity filter of the GluClBr do not involve positively charged residues (as discussed above), then what is the mechanism of Cl− selectivity by the GluClBr? Potential interactions between a Cl− ion and the hydroxyls at positions −3' are also excluded as Thr−3' is deleted in chimeras 13–15, which remain selective to Cl−. The side chain of Ser−4' is likely not involved since it is common to cationic Cys-loop receptors as well.

The determination of relative permselectivities to isethionate and acetate reveals that, although the GluClBr’s pore is not permeable to isethionate, it is slightly permeable to acetate (~10% of the Cl− permeability level). Taking into account the second largest dimension of these two organic anions (Table 2, footnotes), it is reasonable to conclude that the effective open pore diameter of the GluClBr is larger than 5.18 Å and smaller than 6.2 Å. These dimensions fall within the range of the effective open pore diameter determined previously for other anionic Cys-loop receptors such as the GHy- and GABA-activated channels (5.2–6.1 Å (35–38). Given that the Cl−H−O(water) hydrogen bond can be as short as ~2.5 Å, Cl− can snugly fit in the selectivity filter of the GluClBr with one equatorially bound water molecule, as predicted by the structural model (Fig. 5). As such, ion-dipole interactions can potentially take place between a Cl− ion and the backbone amides of positions −2' (but see below) and −3'. In contrast, a Na+ ion would stay outside the selectivity filter where its energy is lower because: (i) even with an equatorially bound water molecule, it is much smaller than the narrowest part of GluClBr's open pore, and (ii) it would not interact with the positive dipoles of the backbone amides. Mutations that widen the pore (chimeras 13–15) probably introduce slight conformational change in the M1-M2 loop, thereby moving the backbone amides of positions −2' and −3' sideways. As such, the progressive widening of the pore results in increasing leak of Cs+ through the pore, whereas the permeability to Na+ remains effectively zero (chimeras 2 and 12 versus chimeras 13–15).

Our observations that a proline substitution at position −2' (chimera 17) does not essentially change the ion charge selectivity or permeability to acetate give rise to one of the following possibilities. (i) Proline does not change the conformation of the main chain in this mutant, as in the textbook case of a T4-lysozyme-A82P mutant (39), or (ii) proline induces a change in the backbone conformation topologically above position −2'. In any event, proline at position −2' prevents the backbone nitrogen of this position to serve as a hydrogen bond donor or to contribute to Cl−-dipole interactions. Conclusively, the backbone amides of position(s) −3' of the GluClBr actually serve as the predominant sites for partial dehydration of a permeating Cl− ion. In line with the experimental results, structural modeling reveals that an A−2'P mutation in the GluClBr's pore does not essentially change the backbone conformation and does not impede the interaction of Cl− with backbone amides at level −3' (Fig. 5D).

It should be noted that the dipole moment of the M2 helices gives partial positive charge at the helices' amino ends. Taken together, the mechanism underlying Cl− selectivity in the GluClBr involves a narrow open pore diameter, attraction of Cl− by the partial positive charge of the M2 N termini, and the interactions of Cl− with backbone amides, where the Cl− ion undergoes partial dehydration. This mechanism might apply to other Cl−-selective ion channels. Indeed, x-ray crystallography studies have recently shown that dehydrated Cl− ions in the pore of a bacterial CIC channel are stabilized by electrostatic interactions with helix dipoles and by partial charges from backbone amide groups and side-chain hydroxyl groups (40).

The Selectivity Filter Overlaps the Activation Gate—Previous studies by Karlin and colleagues (30, 34) and by Paas et al. (20) have located the activation gate of two different Cys-loop receptors in a constricted part close to the bottom of the pore. Paas et al. (20) further probed gating motions in a Cys-loop chimeric receptor by monitoring the state-dependent accessibility of Zn2+ ions to histidines introduced along the channel pore and alignment of the experimental results with computer-assisted molecular models. Consequently, it has been concluded that channel gating predominantly involves rigid tilting motions of the M2 segments, which widen (open) or narrow (close) the bottom pore constriction (20), where ionic selectivity takes place as well (current and the aforementioned other studies). Such a structural overlap implies that components of the activation gate act as the selectivity filter upon channel opening.

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