OF MOLLUSKS, HOMOLOGY MODELING AND DISCOVERY STUDIO

APPLICATION BRIEF

Hyperekplexia and stiff person syndrome can be caused by defects of human glycine receptors, an anionic ligand-gated ion channel. These receptors are found in all the animal kingdom, activated by a diversity of molecules. Understanding how they work in any species will provide insight for the others. The comparison study between two glutamate-gated chloride channels that present a different pharmacology from C. elegans, a nematode, and Aplysia californica, a mollusk provided us with new structural insight into the mechanism of action of this class of receptor.

INTRODUCTION

Rapid synaptic transmission in the nervous system is mediated by a very large and diverse family of ligand-gated ion channels and impairment of these receptors can trigger multiple diseases.

2-cys-loop receptors are anionic ligand-gated ion channels comprising the glycine receptor[1], its unique representative in vertebrates, and receptors in invertebrates that have been shown to be activated by glutamate[1,2,3,4], or histamine[6,7], or inhibited[8] or gated[9] by protons.

Up to now, invertebrate 2-cys-loop receptors have been cloned and expressed only in ecdysozoa (a superphyla comprised of nematodes and arthropods).

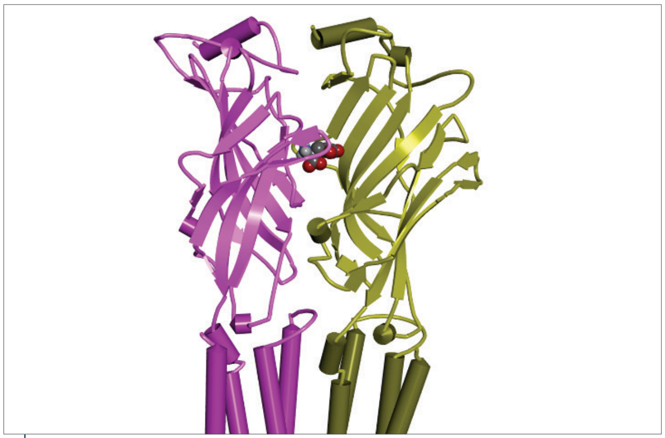

Figure 1: 3D representation of two adjacent subunits of the pentameric glutamate-gated chloride channel in C. elegans as determined by Hibbs and Gouaux. a-helices and B-sheets are respectively represented by tubes and flat arrows. Glutamate at the interface of the two subunits is represented in CPK (PDB code 3RIF).

Success in cloning the first glutamate-gated chloride channels in the lophotrocozoa[10] (the other major superphyla of invertebrates, comprised mainly of molluscs and annelids) along with the determination of the first 3D structure of a glutamate-gated chloride channel in the nematode C. elegans[11] made us envision the possibility of studying new structural determinants of 2-cys-loop receptors and comparing receptors of these two superphylae through molecular modeling.

METHOD

A homology model of the Aplysia californica glutamate-gated chloride channel was built on the basis of the recently published structure of the glutamate-gated chloride channel in C. elegans. As we were interested in the binding of glutamate to these receptors, only two adjacent subunits of the pentameric receptors were considered and modelled.

Principal and complementary faces are respectively displayed in purple and gold in Figure 1.

1. Sequences of the glutamate-gated chloride channels along with other anionic 2-cys-loop receptors were **aligned** using the $Align_{123}$ algorithm[12]:
   - $GluClA_{c1}$ and $GluClA_{c2}$ of Aplysia californica
   - GluClcryst (from the 3D structure) and $GluCl_{a2b}$ (for the biological assays) of C. elegans.
   - the glycine receptor Gly_{a1} from Rattus norvegicus
   - the GABA receptor from Homo sapiens, $GABA_{A-P1}$
2. A **homology model** of $GluClA_{c2}$ was built and refined.
   - The homology model was built based on the abovementioned alignment between $GluClA_{c2}$ and GluClcryst sequences using MODELER[13,14,15]
   - The built homology model was refined using molecular dynamics using CHARMm[16,17].
3. Ligands were **docked** in the homology model of $GluClA_{c2}$: glutamate, B-alanine, y-aminobutyric acid and taurine using CDOCKER[18].
4. Visualization and analysis of the **interactions** between the receptors and the ligands were performed using BIOVIA Discovery Studio.

The biological assays were performed in the Laboratoire de Physiologie Cérébrale at Université Paris Descartes.

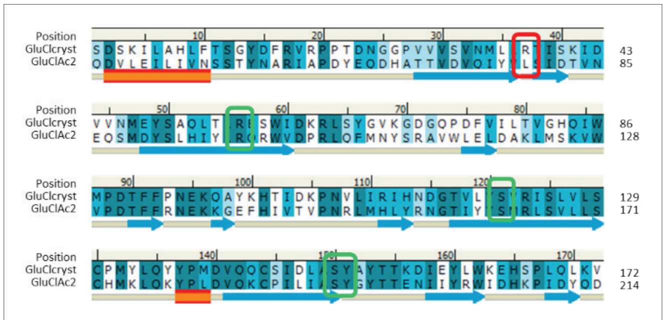

Figure 2: Sequence alignment of the sequences of the extracellular domains of GluClcryst (C. elegans) and GluClA$_{c2}$ (Aplysia californica). Residues binding glutamate in the 3D structure are outlined, in green if an identical or similar residue is aligned in GluClA$_{c2}$ or in red if not.

1. **Site-directed mutagenesis** was performed to obtain mutated receptors. All mutations were confirmed by sequencing.
2. Wild-type and mutated receptors were **expressed** at the surface of Chinese Hamster Ovary cells.
3. **Activity** of the receptors was assessed through electrophysiological recording of individual cells under fast perfusion of solutions containing the considered ligands.

RESULTS

Different pharmacology of C. elegans and Aplysia receptors. While C. elegans glutamate-gated chloride channel GluCl$_{a2b}$ is only activated by glutamate, Aplysia californica GluClA$_{c2}$ is activated by glutamate, but also by B-alanine, GABA and taurine, yet more weakly.

Analysis of the sequence alignment of GluClcryst and GluClA$_{c2}$.

The sequence identity is 34.7%, the sequence similarity is 59.2%. The alignment is compact and all the binding site residues of GluClcryst are conserved in GluClA$_{c2}$ except for R37.

The numbering of the positions used in this document is based on this alignment.

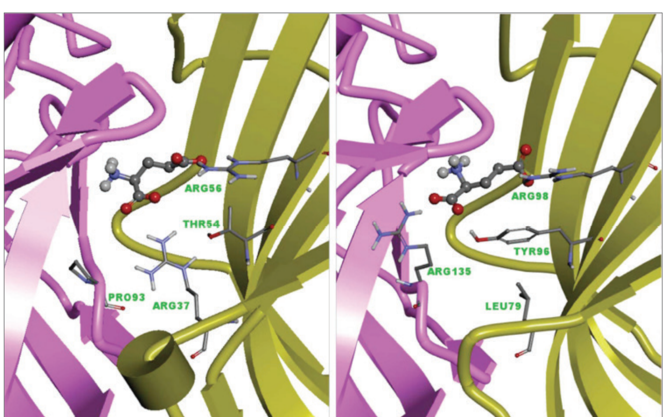

Figure 3: 3D representation of the binding pockets of the 3D structure of GluClcryst on the left, and of the homology model of GluClA$_{c2}$ on the right. Glutamate is displayed in ball and stick and the backbone of the protein in grey ribbon. Residues discussed below are represented as sticks, magenta for the principal face and gold for the complementary face.

Comparison of the binding modes of the two receptors. The binding mode of GluClA$_{c2}$ is predicted thanks to the built homology model.

In both receptors, the γ-carboxylate groups of glutamate are bound to an arginine on the complementary face. The differences between the two binding pockets lie in the binding of the a-carboxylate. In GluClcryst, the a-carboxylate of glutamate is bound to an arginine (R37 identified as a difference in the sequence alignment) on the complementary face, while it is bound to an arginine on the principal face in GluClA$_{c2}$. Moreover, TYR54 in GluClA$_{c2}$ interacts with glutamate and is supported by LEU79, a residue that is aligned with R$_{37}$ in GluClcryst.

Biological assays on mutated receptors.

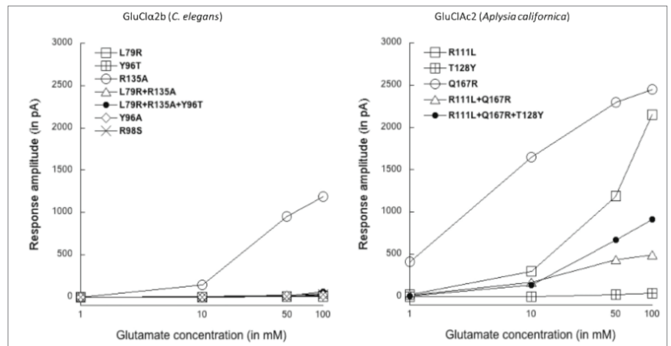

Figure 4: Reponse of mutated receptors to different concentrations of glutamate for C. elegans (left) and Aplysia californica (right).

The role of the residues identified as interacting with the glutamate by the study of the crystallographic structure of GluClcryst and the homology model of GluClA$_{c2}$ have been assessed through biological assays on mutated receptors. The results of mutations at positions suggested by the comparative analysis of the abovementioned structures are shown in Figure 3.

Docking of additional ligands in the homology model of GluClA$_{c2}$.

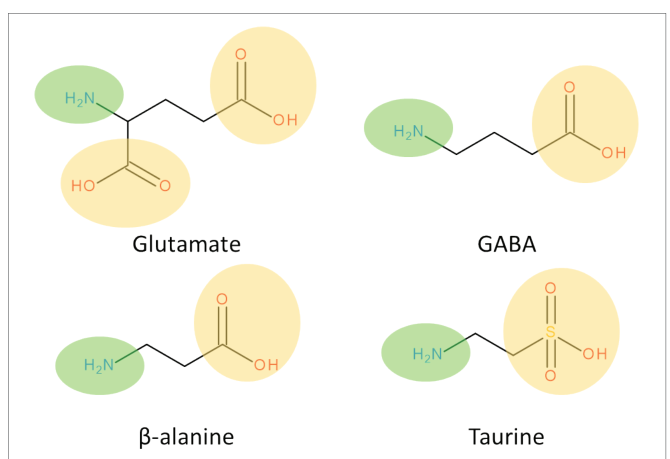

Figure 5: 2D representation of the 4 ligands docked in the homology model of GluClA$_{c2}$. Negatively and positively charged groups at physiological pH are highlighted in orange and green, respectively.

The three ligands present a negatively charged group (carboxylate or sulfonate) and a positively charged group (ammonium) at each side of the carbon chain. Glutamate presents a second carboxylate group in a position.

These ligands adopt similar conformations in their dockings in the homology model of GluClA$_{c2}$. In particular, the negatively charged group on the other end of the carbon chain binds to the same residue: ARG98 (see Figure 3). The ammonium of GABA, B-alanine and taurine is interacting with TYR54 through cation-Pi interaction. Finally, the B-carboxylate of glutamate, that has no equivalent in the other ligands, is interacting with ARG135.

DISCUSSION

Some observations arise when comparing these two glutamate-gated chloride channels in a nematode (C. elegans) and a mollusk (Aplysia californica) using molecular modeling and biological assays, which can be confirmed by the analysis of three other 2-cys-loop receptors.

Pos		GluCl$_{cryst}$	GluClα2b	GluClAc2	GlyRa1	GABA$_A$-ρ	GluClAc1
56	Distal carb. sulfonate	R56	R130	R98	R93	R125	S95
37		R37	R111	L79	N74	E106	L76
54	Proximal carb. (glu)	T54	T128	Y96	F91	Y123	Y93
93		P93	Q167	R135	A129	V161	R132
Glutamate		+	+	+	-	-	-
Other ligands		/	/	β-ala, GABA, tau	gly, GABA, tau	GABA, tau, gly	/

Figure 6: Upper part of the table: list of the aligned residues at the positions of interest in the alignment of 2-cys-loop receptors (position of the residue in the alignment, part of the ligand interacting with the residue, residue for the different receptors). Residues complying to the criteria in the following paragraphs are highlighted in green, the others in red. Lower part of the table: response of each receptor to glutamate and other ligands.

Binding of the ammonium is conserved in all the receptors.

The residues binding the ammonium of all ligands in all receptors is conserved (data not shown). Therefore, the rest of the observations will focus on the differences in binding.

Ligand binding between two subunits of anionic 2-cys-loop receptors requires an arginine at position 56 in the alignment.

As can be seen in Figure 6, the receptors being activated by glutamate or other ligands present an arginine at position 56. This arginine binds the B-carboxylate of glutamate in GluClcryst and GluClA$_{c2}$, and the equivalent negatively charged group of GABA, B-alanine and taurine in GluClA$_{c2}$. The mutation to alanine of the arginines at this position in both GluCl$_{a2b}$ and GluClA$_{c2}$ suppressed all activation by glutamate or other residues in electrophysiology tests (see Figure 4). It is worth noticing that the mutation of this residue in GluCl$_{a2b}$ is the only one for which no response was recovered at higher concentrations of glutamate. Finally, GluClA$_{c1}$ that presents a serine at this position is not activated by glutamate or the other ligands. Therefore, it appears that the interaction with an arginine at position $_{56}$ is essential for ligands to be able to activate the receptor.

A tyrosine or phenylalanine at position 54 favours the ligands without a negatively charged group on Ca of the ligand.

The ammonium groups of GABA, B-alanine and taurine are interacting with Y96 (position 54) in GluClA$_{c2}$. Moreover, LEU79 in GluClA$_{c2}$ (position 37 in the alignment) is supporting TYR96 in the right position (see Figure 3) through van der Waals interactions.

Arginine at position 37 requires a second negatively charged group in the ligand.

GluClcryst and GluCl$_{a2b}$ are activated only by glutamate. As can be seen in Figure 3, the arginine at position 37 in GluClcryst (R37) is located on the complementary face, in the middle of the binding pocket and structurally in the same location as Y54 in GluClA$_{c2}$. It has already been stated that the ammonium groups of GABA, B-alanine and taurine are interacting with Y54 in GluClA$_{c2}$.Therefore, these three ligands in GluClcryst would have their ammonium facing the arginine R37. This would create a repulsion, which is screened by the a-carboxylate of glutamate. Hence, an arginine at position 37 requires a negatively charged group on the ligand to screen the repulsion.

An arginine at position 37 cannot accommodate a phenylalanine or tyrosine at position 54.

It has already been observed that LEU$_{79}$ is supporting TYR96 in GluClA$_{c2}$. Moreover, TYR96 is essential to ligand binding in GluClA$_{c2}$ as it mutation to alanine or threonine suppressed all response of the receptor (see Figure 2). Hence, mutating LEU79 into an arginine should impede TYR96 to be rightly placed in the binding pocket. Indeed, both mutating LEU79 into an arginine in GluClA$_{c2}$ and mutating THR54 into a tyrosine in GluCla2b suppressed activation of the receptors.

An arginine at position 93 favours the binding of glutamate.

It has been already observed that a tyrosine or a phenylalanine at position 54 cannot accommodate an arginine at position 37. The a-carboxylate group of glutamate in receptors having a tyrosine or phenylalanine at position 54 can interact with an arginine at position 93 (see Figure 3). Indeed, GluClA$_{c2}$ presents an arginine at this position (R135) and is activated by glutamate, while GlyR$_{a1}$ and GABA$_{A\text{-}p1}$ do not present this arginine and are not activated by glutamate.

CONCLUSIONS

Thanks to the combined results of homology modeling using BIOVIA Discovery Studio and experimental biology tests, some structural determinants of 2-cys-loop receptors have been discovered. Moreover, the ligand specificity of these 2-cys-loop receptors has been reduced to a simple analysis of residue composition at essential positions in the sequence alignment with other 2-cys-loop receptors. With this new tool in hand, the ligand specificity of an orphan 2-cys-loop receptor can be predicted and therefore help to characterize it. Finally, the structural proximity between the Aplysia californica glutamate-gated chloride channel and the human glycine receptor can envision the possibility to use the former as an experimental model to study the latter.

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