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Binding Modes of Noncompetitive GABA-Channel Blockers Revisited Using Engineered Affinity-Labeling Reactions Combined with New Docking Studies†

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ABSTRACT: The binding modes of noncompetitive $GABA_A$-channel blockers were re-examined taking into account the recent description of the 3D structure of prokaryotic pentameric ligand-gated ion channels, which provided access to new mammalian or insect GABA receptor models, emphasizing their transmembrane portion. Two putative binding modes were deciphered for this class of compounds, including the insecticide fipronil, located nearby either the intra- or the extracellular part of the membrane, respectively. These results are in full agreement with previously described affinity-labeling reactions performed with $GABA_A$ noncompetitive blockers (Perret et al. *J. Biol. Chem.* **1999**, *274*, 25350−25354).

KEYWORDS: fipronil binding site, $GABA_A$ receptor models

INTRODUCTION

Despite recent remarkable successes in the resolution of membrane receptors' structure by X-ray crystallography, the deciphering of a complete 3D structure of eukaryotic ligand-gated ion channels (LGICs) remains a true challenge in structural biology,[1,2] and as a consequence the structure of complete LGIC has never been obtained in the presence of an agonist. In the absence of such data, ligand receptor interaction studies are typically investigated by means of site-directed approaches either through site-directed mutagenesis or by site-directed labeling reactions, that is, affinity or photoaffinity labeling. Both approaches, in an interactive fashion, use homology models built from X-ray data of a cognate protein such as the acetylcholine binding protein (AChBP),[3,4] a pentameric architecture that has been taken as a surrogate for the extracellular portion of the nicotinic acetylcholine receptor (nAChR)[5] or for the GABA receptor.[6]

Site-directed mutagenesis, although generating important information on receptor structure function interplay, will always be subject to a fundamental questioning of its true relevance due to possible structural allosteric modifications. Alternatively, photoaffinity labeling[7] performed on native receptors or on the surrogate receptor[8] does, however, require fairly large amounts of homogeneous purified receptor and therefore cannot be used for the labeling of pertinent neuronal receptor or receptor subtypes. This situation prompted us to develop an alternative engineered affinity labeling methodology, which can be applied to any receptor subtype expressed in usual cellular systems and does use electrophysiology for its analysis.[9] This method covalently and selectively links thiol-reactive probes to single engineered cysteines in receptor binding sites selected after receptor ligand docking models. Importantly, these mutant receptors are controlled to remain fully functional to circumvent the above-mentioned caveat. The demonstration of a specific covalent bond formation allows an apposition of the cysteine side chain with the reactive moiety of the probe and a correct orientation of the probe in the receptor binding site thereof. Several successful examples on the interaction of a series of ligands with their receptors could be demonstrated using this methodology[10,11] including the interaction of noncompetitive channel blockers (NCAs) (See Supporting Information Figure 1), notably the insecticide fipronil on the GABA-gated chloride channel,[12] known to interact at the transmembrane pore constituted by the M2-helices of the pentameric $GABA_A$ receptor. Structural models for the binding of several major insecticides were determined using site-directed mutagenesis, binding studies, and molecular modeling,[13,14] whereas homology modeling of human $\alpha1\beta1\gamma2$ and housefly $\beta3$ GABA receptors was built using the low-resolution cryoelectron microscopic structure of the torpedo nAChR as a template.[15] Although the involvement of the cytoplasmic half of the pentameric M2-helix pore for the binding of the different NCAs seems to be in agreement with most published data, its exact position within the pore, in particular for the fipronil molecule, remains controversial.[14,15]

The recent description of the X-ray structure of prokaryotic pentameric ligand gated ion channel[16−18] provided an important model system for the functioning of ligand gated ion channel

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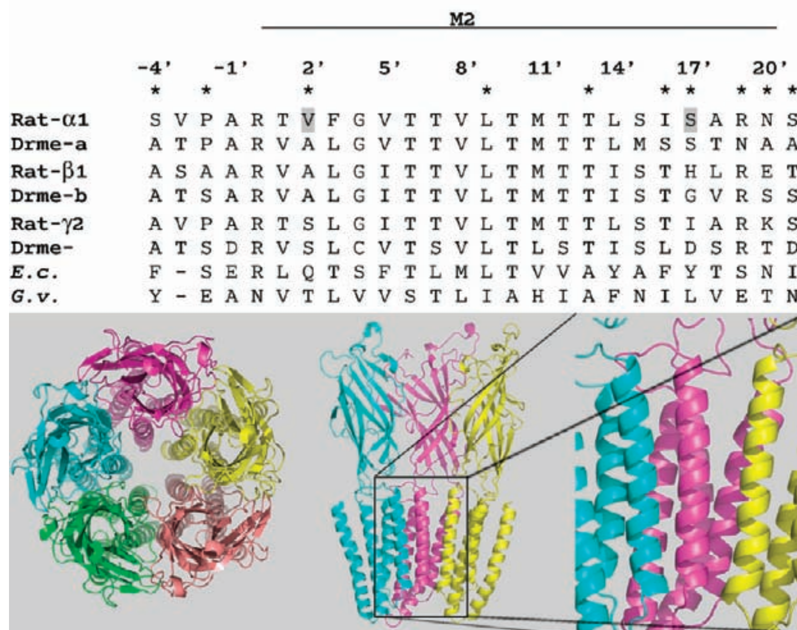

Figure 1. Model preparation. (Top) Sequence alignment at the level of the M2 helix. Stars highlight residues of the model that are solvent-accessible (see Supporting Information, Figure 2). (Bottom) Rat α1β2γ2 receptor homology model: (left) complete pentamer viewed from the extracellular space; (middle) three subunits viewed from the ion channel; (right) close-up view of three M2 helices lining the ion channel.

receptors and represents significant advances in the structural knowledge of the nicotinic receptor and the LGICs thereof.[19] In particular, the new information on the transmembrane part of these receptors opens the way for a better understanding of the interaction of noncompetitive channel blockers with their receptors. In the present paper, we have built new models of the rat α1β2γ2 receptor based on the prokaryotic pentameric LGIC. These models were then used in docking experiments to study the binding modes of the above-mentioned chemical probes. In agreement with previously determined labeling experiments[12] two putative binding modes were found in the ion channel for the studied noncompetitive channel blockers.

MATERIALS AND METHODS

Homology Modeling. Sequences of insect and mammalian GABA-R and of mammalian nAChR were retrieved from the LGICdb.[20] The large number of sequences used is intended to overcome the difficulty arising from the low sequence conservation with prokaryotic homologues (see, for example, Figure 1A). A multiple alignment of these sequences together with those of the prokaryotic LGIC available in the PDB was prepared with T-Coffee.[21]

The aligned sequences of rat and prokaryotic LGIC were used for homology modeling with Modeler 9v7.[22] The template structures were those of *Gloeobacter violaceus* (PDBid: 3EAM) and *Erwinia crysantemi* (PDBid: 2VL0). The helical character of M1 was enforced using special restrains. For each template 10 models were first generated and then 4 attempts of loop optimization were made for each model. The resulting models with optimized loops were evaluated with the DOPE energy function, and the best one was kept.

Ligand Structure File Preparation. Structures of fipronil, picrotoxin, and EBOB were retrieved as 3D .sdf files from Pubchem[23] and converted into pdb files using OpenBabel.[24] Bromofipronil was modified from fipronil using Molden 4.8.[25]

Docking. Receptor and ligand pdbqt files preparation was conducted with Autodock tools.[26] The docking was performed with the software Autodock vina.[27] Three boxes, 30 Å on side, were constructed: one around each α1−272 at the upper extracellular side of the ion channel and one at the intracellular side of the ion channel encompassing both α1−257. The boxes, which partially overlap, cover the full ion channel. The docking was performed with default settings except that it was repeated 100 times, leading to up to 20 clusters. The resulting docking poses were analyzed with Pymol[28] to detect poses in which the chemical compound is in the proximity of the experimentally identified residues.

To validate the docking poses obtained by Autodock, we used a second docking program based, as Autodock vina, on a genetic algorithm (GA). GOLD program (v 4.1.2)[29] was used with default settings for the GA parameters. To allow weak nonbonded contacts at the start of each GA run, the maximum distance between hydrogen donors and fitting points was set to 5 Å. To further speed the calculation, the GA docking was stopped when the top three solutions were within 1.5 Å root-mean-square deviation. It can be assumed when the criterion is met that these top solutions represent a reproducible pose for the ligand. A spatial constraint was used to force match to experimental data.

RESULTS AND DISCUSSION

Molecular Models of Rat GABA Receptors. Two models of the rat α1β1γ2 $GABA_A$ receptor, used in the affinity-labeling study,[12] were prepared by comparative modeling (see Figure 1 and Materials and Methods). The templates are the prokaryotic homologues from *G. violaceus* solved in the active state[17,18] and from *E. chrysanthemi* solved in a closed state.[16] As expected, the resulting models display an open and a closed ion channel, respectively.

Site-directed labeling experiments of the NCA binding site of the rat α1β1γ2 $GABA_A$ receptor containing engineered cysteines at the M2 transmembrane helix identified two positions, α1−Val257 and α1−Ser272, respectively, for specific covalent bond formation with cysteine-reactive NCAs including a bromofipronil derivative.[12] The latter position is homologous to the

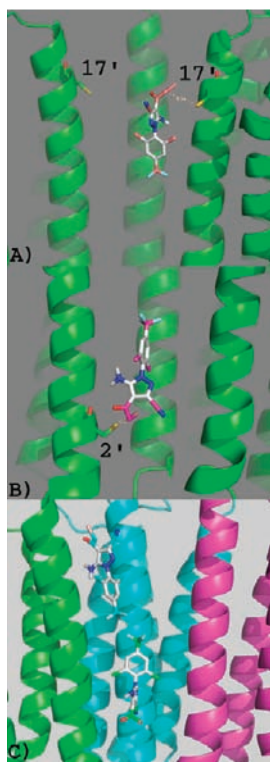

Figure 2. Binding mode of the cysteine-reactive compound bromofipronil obtained by docking on the rat $\alpha1\beta2\gamma2$ receptor model for the mutant S272C (A) or V257C (B) and the wild type (C). The view is similar to the bottom-right view of Figure 1. The protein and bromofipronil are shown in cartoon and stick representations, respectively.

residue β1−His292 found to participate in the Zn^{2+} binding site.[30,31] We prepared models of the α1−V257C and α1−S272C mutants for docking.

Docking. The docking study presented here has been initiated to challenge as accurately as possible the experimental affinity-labeling data. Therefore, binding modes were selected to display a minimal distance between the reactive moieties of the probe with the side chain of the experimentally identified residues. Interestingly, similar binding modes were found with both docking softwares used (Autodock vina and GOLD), leading therefore to robust results.

All compounds were tentatively docked on both open- and closed-ion channels models. However, binding poses in agreement with experiments were found only for the open-ion channel model. Docking with the closed-ion channel model allowed the approach of bromofipronil toward C272 (on the α1−S272C mutant) but not to C257 (on the α1−V257C mutant). These results do not fit the experimental data and therefore the following docking experiments were performed on the open-ion channel model.

It is noteworthy that the docking is capable of reproducing the experimental data in the open-channel model but not in the closed-channel model. It implies that bromofipronil and, therefore, fipronil are open-channel blockers. This specificity of the compounds was not addressed originally for bromofipronil[12] but was later proposed for fipronil.[32]

Affinity-Labeling Probe Bromofipronil. The affinity-labeling probe bromofipronil was first docked on the two cysteine mutants for which affinity labeling was measured[12] using the software Autodock vina.[27] Solutions compatible with a reaction were found for both of the two mutated positions (Figure 2A,B). Their computed energies are −6.4 and −5.5 kcal mol^{-1} for the lower site (facing V257C) and for the upper site (facing S272C), respectively. These binding poses were also reproduced with the software GOLD (Supporting Information, Figure 3). The distances between the reactive carbon and the Cys side chain are 4.3 and 3.3 Å for S272C and 4.5 and 3.6 Å for V257C, for Autodock and GOLD, respectively. Therefore, in both solutions (Autodock vina and GOLD) these distances were compatible with an affinity-labeling reaction.

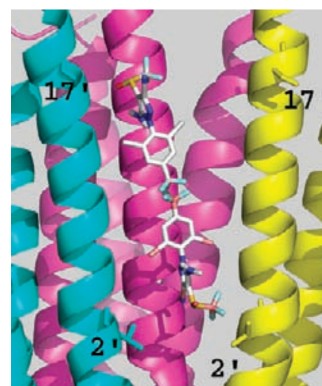

Figure 3. Binding modes of the insecticide fipronil obtained by docking on the rat WT-$\alpha1\beta2\gamma2$ receptor model. The two binding modes were obtained independently. The view is similar to the bottom-right view of Figure 1. The protein and fipronil are shown in cartoon and stick representations, respectively.

Bromofipronil was then docked to the wild type receptor to test whether or not the two binding modes found on the mutants are also accessible in the wild type. Interestingly, these binding modes were found to be reachable by Autodock vina (Figure 2C) and were also found with GOLD.

Fipronil. The docking of the insecticide fipronil was performed on the rat WT-$\alpha1\beta1\gamma2$ $GABA_A$ receptor. Binding modes similar to those obtained with bromofipronil were found to be favorable (Figure 3). Their computed energies are −6.8 and −5.5 kcal mol^{-1} for the lower and upper site, respectively. Both binding modes are reproduced with GOLD provided that a distance constraint to the residue is used.

Picrotoxin. The docking of the ion channel blocker picrotoxin was performed on the rat WT-$\alpha1\beta1\gamma2$ $GABA_A$ receptor and disclosed the existence of two binding sites, one at the cytoplasmic end of the ion channel and the other at the extracellular end (Figure 4). Their computed energies are −7.5 and −6.6 kcal mol^{-1} for the lower and upper site, respectively. The two sites overlap with those of bromofipronil (Figure 4) in agreement with the experimental observation that picrotoxin can prevent the covalent reaction of bromofipronil on the cysteine mutants.[12]

Ethynylbicycloorthobenzoate (EBOB). The docking of the ion channel blocker EBOB was performed on the rat WT-$\alpha1\beta1\gamma2$ $GABA_A$ receptor revealing again two binding modes (Figure 5). Their computed energies are −6.3 and −6.4 kcal mol^{-1} for the lower and upper site, respectively. One of them at the intracellular end of the ion channel appears to be similar to previous findings.[14]

General Observations about the NCA Binding Sites. Two binding sites for bromofipronil were observed in the ion channel, in agreement with the labeling experiments. Two sites were also

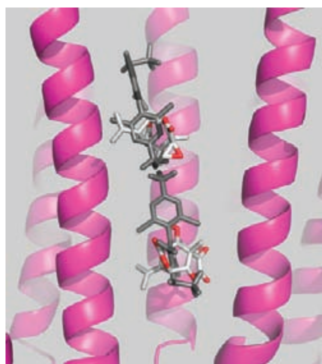

Figure 4. Binding modes of the channel blocker picrotoxin obtained by docking on the rat WT-$\alpha1\beta2\gamma2$ receptor model. The two binding modes are obtained independently and are overlaid with the binding modes of fipronil (dark gray). The view is similar to the bottom-right view of Figure 1. The protein and picrotoxin/fipronil are shown in cartoon and stick representations, respectively.

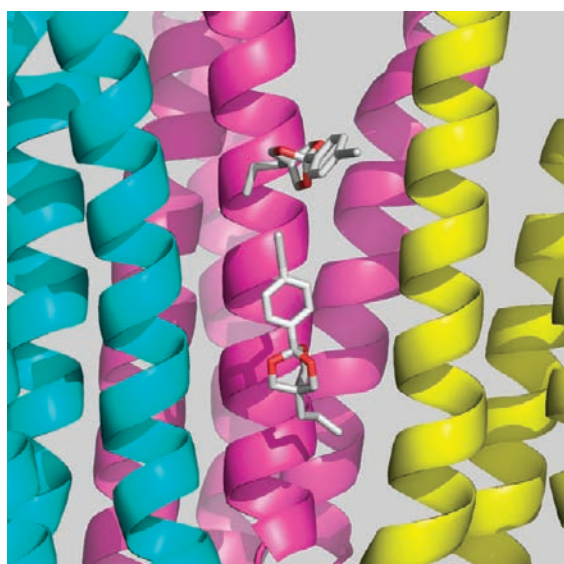

Figure 5. Binding modes of the insecticide ethynylbicycloorthobenzoate (EBOB) obtained by docking on the rat WT-$\alpha1\beta2\gamma2$ receptor model. The two binding modes were obtained independently. The view is similar to the bottom-right view of Figure 1. The protein and EBOB are shown in cartoon and stick representations, respectively.

observed for the other compounds tested, fipronil, picrotoxin, and EBOB, respectively, although these results are less robust as indicated by the difficulty of reproducing them with GOLD. The lower site always corresponds to the lowest energy pose, and the upper site has a higher energy except for EBOB, for which both are similar. Nevertheless, it may be proposed that the two sites are also accessible to these NCAs. In addition, it is found that, when bromofipronil, picrotoxin, and EBOB are positioned in both sites, there is no steric collision, so the two sites are probably not mutually exclusive, and therefore it can be proposed that they could be occupied simultaneously.

The two sites illustrated here involve the central, hydrophobic, region of the ion channel,[19] which may explain the capacity to bind diverse compounds in a relatively unspecific way.[14] This is also reminiscent of the observation that some neonicotinoids can adopt two bound conformations in the extracellular domain of a vertebrate receptor model, which are head to foot.[33] The occurrence of two complementary binding sites might offer in the future the possibility to design new insecticides with potentially increased affinity and specificity, that is, by connecting covalently tail-to-tail two molecules of fipronil.

ASSOCIATED CONTENT

Supporting Information. Additional figures. This material is available free of charge via the Internet at http://pubs.acs.org.

Present Addresses

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