Molecular Determinants of Ivermectin Sensitivity at the Glycine Receptor Chloride Channel*

Received for publication, May 19, 2011, and in revised form, October 25, 2011 Published, JBC Papers in Press, October 27, 2011 DOI 10.1074/jbc.M111.262634

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Background: The ivermectin-binding site on the glutamate-gated chloride channel was recently resolved by crystallography.

Results: Ivermectin binds in a similar orientation to the structurally related glycine receptor, although two H-bonds apparent in the crystal structure proved unimportant for binding to glycine receptors.

Conclusion: Ivermectin-binding mechanisms vary among Cys-loop receptors.

Significance: Understanding ivermectin-binding mechanisms may help in designing new drugs.

Ivermectin is an anthelmintic drug that works by activating glutamate-gated chloride channel receptors (GluClRs) in nematode parasites. GluClRs belong to the Cys-loop receptor family that also includes glycine receptor (GlyR) chloride channels. GluClRs and A288G mutant GlyRs are both activated by low nanomolar ivermectin concentrations. The crystal structure of the Caenorhabditis elegans α GluClR complexed with ivermectin has recently been published. Here, we probed ivermectin sensitivity determinants on the α1 GlyR using site-directed mutagenesis and electrophysiology. Based on a mutagenesis screen of transmembrane residues, we identified Ala288 and Pro230 as crucial sensitivity determinants. A comparison of the actions of selamectin and ivermectin suggested the benzofuran C05-OH was required for high efficacy. When taken together with docking simulations, these results supported a GlyR ivermectin binding orientation similar to that seen in the GluClR crystal structure. However, whereas the crystal structure shows that ivermectin interacts with the α GluClR via H-bonds with Leu218, Ser260, and Thr285 (α GluClR numbering), our data indicate that H-bonds with residues homologous to Ser260 and Thr285 are not important for high ivermectin sensitivity or direct agonist efficacy in A288G α1 GlyRs or three other GluClRs. Our data also suggest that van der Waals interactions between the ivermectin disaccharide and GlyR M2–M3 loop residues are unimportant for high ivermectin sensitivity. Thus, although our results corroborate the ivermectin binding orientation as revealed by the crystal structure, they demonstrate that some of the binding interactions revealed by this structure do not pertain to other highly ivermectin-sensitive Cys-loop receptors.

Ivermectin is a semi-synthetic anthelmintic drug used widely in human medicine and veterinary practice (1). The biological target for ivermectin and related macrocyclic lactones is a glutamate-gated chloride channel receptor (GluClR) that is expressed in the neurons and muscle cells of nematodes and some arthropods but is absent in vertebrates (2). Ivermectin irreversibly activates these GluClRs at low nanomolar concentrations, thereby inhibiting neuronal activity and muscle contractility and thus inducing death by flaccid paralysis (3). Unfortunately, ivermectin resistance is emerging as a serious problem in nematodes and arthropods (4–6). In several instances, resistance has been shown to be caused by mutations that reduce the GluClR ivermectin sensitivity (7–9). Insight into the binding mechanisms of ivermectin at the GluClR may contribute to the understanding of ivermectin resistance mechanisms and to the development of a much needed new generation of anthelmintic drugs. A 3.3-Å crystal structure of the Caenorhabditis elegans α GluClR with ivermectin bound has recently been published (10), revealing ivermectin’s molecular interactions at atomic resolution.

GluClRs belong to the Cys-loop receptor superfamily that also includes the excitatory nicotinic acetylcholine (nAChR) and 5-hydroxytryptamine type 3 receptors. The inhibitory γ-aminobutyric acid type A receptor (GABAAR), and the inhibitory GlyR. Cys-loop receptors are formed by five homologous subunits that each consist of an N-terminal ligand-binding domain (LBD) and a bundle of four transmembrane helices (M1–M4) that constitute the transmembrane domain (TMD). M2 helices contributed from each subunit line the central ion channel pore. Neurotransmitter ligand-binding sites lie at the interface of LBDs of adjacent subunits.

Ivermectin also interacts with many vertebrate Cys-loop receptors but usually only at high (micromolar) concentrations. For example, GABAARs and GlyRs are directly activated by ivermectin at 1–2 μM (11, 12), and acetylcholine-induced cur-

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* This work was supported in part by the Australian Research Council and the National Health and Medical Research Council of Australia.

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5 The abbreviations used are: GluClR, glutamate-gated chloride channel receptor; LBD, extracellular domain; GABAAR, GABA type A receptor; GlyR, glycine receptor; nAChR, nicotinic acetylcholine receptor; pre-M1, the domain preceding the first transmembrane segment; TMD, transmembrane domain.
Glycine Receptor Ivermectin-binding Site

rents at α7 nAChRs are potentiated by a pre-application of 30 μM ivermectin (13, 14). Insight into the binding mechanisms of ivermectin at human Cys-loop receptors may contribute to the characterization of novel therapeutic pharmacophores. For this reason, we sought to identify the molecular basis of ivermectin binding to the α1 GlyR.

The α GluClR-ivermectin crystal structure (10) was published after the experiments described in Figs. 1–7 were completed. With prior knowledge of the crystal structure, our experimental design would have been different. However, because all of our data remain relevant, we describe our original experiments in the context of our original experimental design. Following this, we generate a structural model of the α1 GlyR ivermectin-binding site on the basis of our data, compare it with the crystal structure binding site, and then experimentally verify whether it can account for ivermectin binding to the α1 GlyR.

EXPERIMENTAL PROCEDURES

Molecular Biology—The human α1 GlyR subunit and the Hemonchus contortus GluClR α3B subunit cDNAs were subcloned into the pCIS and pcDNA3.1 plasmid vectors, respectively. Site-directed mutagenesis was performed using the QuickChange mutagenesis kit (Stratagene, La Jolla, CA), and the cloned into the pCIS and pcDNA3.1 plasmid vectors, respectively. Site-directed mutagenesis was performed using the QuickChange mutagenesis kit (Stratagene, La Jolla, CA), and the successful incorporation of mutations was confirmed by DNA sequencing. HEK-293 Cell Culture and Transfection—HEK-293 cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) containing Serum Supreme (Lonza, Walkersville, MD) and penicillin/streptomycin, dissolved in water stored as 10 mM stocks at −20 °C. Thus, solutions containing 30 μM ivermectin (the highest concentration routinely used) also contained 0.3% dimethyl sulfoxide. This concentration of dimethyl sulfoxide showed no effects on the membrane resistance of cells. Solutions were applied to cells via gravity-induced perfusion systems fabricated from polyethylene tubing. All experiments were performed at room temperature (19–22 °C). Agonist dose-response experiments were performed as described below. For each dose-response experiment, the half-maximal agonist concentration (EC50), Hill coefficient (nH), and saturating agonist current magnitude (Imax) values were determined by fitting individual dose-response relationships with the three-parameter Hill equation (SigmaPlot® 11, Jandel Scientific, San Rafael, CA). Results are expressed as mean ± S.E. from at least three experiments. Unpaired t tests (SigmaPlot® 11, Jandel Scientific, San Rafael, CA) were used to compare these values, as described in the table legends.

GlyR Structural Modeling and Computational Docking—A homology model of the α1 GlyR pentamer was built based on a hybrid template, with the TMD and Cys-loop based on the bacterial ELIC channel structure (Protein Data Bank code 2VL0) (15) and the remainder of the LBD based on acetylcholine-binding protein (Protein Data Bank code 119B) (16), as described previously (17). Two distinctly different families of ivermectin conformers were predicted by MarvinSketch software (Chemaxon, Budapest, Hungary). AutoDock Vina (18) was used to explore feasible interactions of each of these two conformer families with WT and A288G mutant model GlyRs, within a 40 × 40 × 30-Å box surrounding the Ala288 residue. Conformer 1 gave consistently stronger binding energies than conformer 2 in all docking experiments, so only the results for conformer 1 are presented here.

Following the publication of the α GluClR-ivermectin crystal structure (10), in which the bound ivermectin was in the conformer 1 family, we built a new homology model of the α1 GlyR pentamer based on this structure (Protein Data Bank code 3RIF). Ivermectin in conformer 1 was then docked to this model, as described above. As a control, equivalent docking was carried out on 3RIF from which the bound ivermectin had been removed prior to docking.

RESULTS

Disruption of Ivermectin Efficacy via Mutations at the LBD-TMD Interface—Because the conservative Y279F mutation in the α1 GlyR M2–M3 loop dramatically reduces ivermectin sensitivity (19), we investigated whether residues at the LBD-TMD interfacial region may contribute to ivermectin-binding or -gating mechanisms. We thus introduced cysteines one at a time for each residue from R271C (19) to I283C. Each mutant GlyR was expressed in HEK-293 cells via calcium phosphate method with the pcDNA3.1 plasmid vector (Clontech) as a fluorescent transfection marker. Typically, 250 ng of each plasmid was used to transfect each 3-cm dish. After 12–18 h in the transfection medium, cells were washed twice with calcium-free phosphate-buffered saline and returned to DMEM. Cells were used in experiments 24–48 h later.

Electrophysiology and Data Analysis—An inverted fluorescence microscope was used to visualize cells for electrophysiological experiments. Cells expressing recombinant GluClRs or GlyRs were identified by their green fluorescence. Borosilicate glass capillary tubes (Vitrex, Modulohm, Denmark) and a horizontal pipette puller (P97, Sutter Instruments, Novato, CA) were used to pull patch clamp pipettes with tip resistances of 2–3 megohms when filled with pipette solution consisting of (in mM) 145 CsCl, 2 CaCl2, 2 MgCl2, 10 HEPES, and 10 EGTA, adjusted to pH 7.4 with 2 M NaOH. Drug solutions were prepared from at least three experiments. Unpaired t tests (SigmaPlot® 11, Jandel Scientific, San Rafael, CA) were used to compare these values, as described in the table legends.

Mean glycine EC50 values and peak current magnitudes of all mutant receptors have previously been reported (20). As shown in Fig. 1A, most mutant GlyRs showed little or
no activation at 0.3 μM ivermectin but strong activation at 30 μM ivermectin. Apart from Y279F, P275C was the only mutation that significantly reduced the magnitude of the current activated by 3 μM ivermectin relative to the corresponding value observed at the WT GlyR. The locations of interfacial residues influencing ivermectin efficacy. Two orthogonal views of the interfacial region are shown. C, sample ivermectin dose-response relationships at the WT, Y279F, and L142C GlyRs. In this and all subsequent figures, glycine and ivermectin applications are shown as filled and open bars, respectively.

The locations of Pro275 and Tyr279 in an α1 GlyR structural homology model are shown in Fig. 1B. The averaged ivermectin activation dose-response relationships for both receptors are shown in Fig. 1E with mean parameters of best fit summarized in Table 1. Both mutations dramatically reduced receptor sensitivity to both ivermectin and glycine (Tables 1 and 2). Unlike Y279F, the Y279C mutation had little effect on ivermectin sensitivity (Table 1) but produced a large rightward shift in the glycine EC50 (19, 20).

We next investigated possible ivermectin sensitivity determinants in the LBD regions (i.e. loop 2, conserved Cys-loop pre-M1 domain) that lie proximal to Pro275 and Tyr279 in our model α1 GlyR structure. In loop 2, we investigated A52C and E53C not only because of their proximity to Tyr279 but also because of the known role of A52 as an alcohol sensitivity determinant (22, 23). We also investigated D141G, L142C, and...
K143C in the conserved Cys-loop and Y222C and Y223C in the pre-M1 domain. Ivermectin dose responses were quantitated for all mutant GlyRs, with sample ivermectin-induced currents for the L142C mutant GlyR shown in Fig. 1C. Averaged parameters of best fit for this and all other mutant GlyRs are summarized in Table 1. The L142C and Y222C mutations dramatically reduced receptor sensitivity to both ivermectin and glycine (Tables 1 and 2 and Fig. 1E). Given this nonselective action on glycine- and ivermectin-mediated currents, and the locations of these mutations in known agonist transduction pathways (24), we hypothesized that these mutations disrupted the ivermectin gating efficacy. To test this, we quantitated the potency with which ivermectin potentiated glycine-activated currents. This was performed by alternating low (EC$_{50}$–EC$_{10}$) glycine applications with 5-s applications of increasing ivermectin concentrations (Fig. 1D). Averaged ivermectin potentiating dose-response relationships for the WT, L142C, Y222C, P275C, and Y279F GlyRs reveal that the potentiating effects of ivermectin occurred at lower concentrations at these mutant GlyRs than at the WT GlyR (Fig. 1F and Table 3). Together, these results indicate that the L142C, Y222C, P275C, and Y279F substitutions disrupt ivermectin efficacy while having little effect on its affinity.

We then tested the effects of these mutations on the highly ivermectin-sensitive A288G mutant GlyR (21). As summarized in Table 4, the A288G mutation adds to the deleterious effect of these mutations on ivermectin efficacy. Identification of TMD Residues That Influence Ivermectin Sensitivity—We have already shown that the A288F mutation eliminates ivermectin agonist sensitivity (25). However, it has yet to be investigated whether this mutation also affects the sensitivity with which ivermectin potentiates glycine currents. Using a protocol as described in Fig. 1D, we observed significant potentiating of glycine-gated currents only at ivermectin concentrations >10 µM (Fig. 2, A and B). This is consistent with A288F disrupting an ivermectin-binding site.

As discussed below, our GlyR model places Ala$^{288}$ near the extracellular end of M3, facing across the subunit interface toward M1 of the adjacent subunit (26–29). From our ELIC-based model, we identified 10 residues as having side chains close to or directed toward Ala$^{288}$ as follows: six in the M1 domain (Ile$^{225}$, Gln$^{226}$, Ile$^{229}$, Pro$^{230}$, Leu$^{233}$, and Ile$^{234}$) and three M2 side chains that face away from the channel pore (Val$^{266}$, Thr$^{266}$, and Gln$^{266}$ which correspond to the 8’, 12’, and 14’, positions, respectively) and an M3 residue, Leu$^{291}$, located one helical turn below Ala$^{288}$. We individually mutated each of these residues to tryptophan, on the grounds that the bulky tryptophan side chain may occlude access to a putative ivermectin-binding site in the vicinity of Ala$^{288}$. Tryptophan substitution has been employed at GABA$\_\alpha$Rs to elucidate transmembrane domain binding sites for alcohols and anesthetics.
Glycine Receptor Ivermectin-binding Site

Mean ivermectin dose-response relationships for these mutants are plotted in Fig. 3, A and B (right panels), with averaged parameters of best fit summarized in Table 1. Sample ivermectin dose-response relationships for the I229W, P230W, and L233W mutant GlyRs are shown in Fig. 3C. Most mutants did not differ significantly from WT in their ivermectin sensitivity. However, the P230W GlyR exhibited a significantly increased ivermectin EC\textsubscript{50} value and a significantly reduced \(I_{\text{max}}\) value relative to the WT GlyR value (Table 1). Also, at the Q226W mutant GlyR, maximal ivermectin-activated currents were significantly larger than the WT GlyR value, although ivermectin sensitivity was significantly decreased (Table 1). These results implicate Glu\textsuperscript{226} and Pro\textsuperscript{236} as ivermectin sensitivity determinants. The L233W, L291W, and T264W mutant GlyRs were not activated by ivermectin at concentrations of up to 100 \(\mu\text{M}\) (Fig. 3C and Table 1).

At the L233W and L291W mutant GlyRs, we noticed that responses to saturating glycine were reduced in magnitude or even absent when glycine was applied after ivermectin (e.g. Fig. 3C). At both mutant GlyRs, a 5-s application of 10 \(\mu\text{M}\) ivermectin caused a significant (\(p < 0.05\)) decrease in the time taken for glycine-activated current to decay to half-maximum amplitude (Fig. 4, A and B). In addition, subsequent glycine-activated currents were dramatically decreased in magnitude (Fig. 4A). This effect of ivermectin proved to be irreversible. Although single applications of 1 or 3 \(\mu\text{M}\) ivermectin had little effect on peak glycine-activated current magnitude, repeated applications of 1 \(\mu\text{M}\) ivermectin produced a slowly developing but consistent increase in the desensitization rate (data not shown). Unfortunately, the slow onset and irreversibility of this effect made it difficult to quantitate its ivermectin sensitivity. To determine whether Leu\textsuperscript{233} and Leu\textsuperscript{291} were specifically required for ivermectin to exert an agonist effect, we investigated the effects of ivermectin on the highly nonconservative L233Q and L291Q mutant GlyRs. As summarized in Table 1, ivermectin activated both receptors with \(EC_{50}\) and \(I_{\text{max}}\) values that were not significantly different from WT values. Thus, the desensitization-enhancing effect of ivermectin appears to require bulky tryptophan side chains at either of the two sites. In the model presented below, these two residues lie opposite each other across the intersubunit interface.

The T264W (12\textsuperscript{'}\) mutant GlyR responded in an unusual manner to both glycine and ivermectin. Glycine activated a transient inward current that was followed by a distinct upward deflection that persisted for the duration of the glycine application (Fig. 4C). Consistent with a previous study that observed a similar phenomenon in mutant homomeric \(\rho1\) GABA\textsubscript{A}Rs (35), we speculate that prolonged glycine application inhibited a leak current through these receptors. Ivermectin produced an irreversible dose-dependent inhibition of this leak current (Fig. 4C), with a mean IC\textsubscript{50} of 2.5 ± 1.4 \(\mu\text{M}\) and an \(n_I\) value of 1.3 ± 0.2 (both \(n = 4\)). This IC\textsubscript{50} value, which is not significantly different from the WT value, suggests that ivermectin sensitivity is not dramatically affected by the mutation.

We next tested whether the M1 domain tryptophan substitutions impaired ivermectin sensitivity in the A288G mutant

\[I_{\text{max}}\text{ were observed at both the V260W and T264W GlyRs (Table 2).}\]

\[\text{TABLE 4}\]

Direct agonist effects of ivermectin on GlyR mutants incorporating A288G

| Receptor     | EC\textsubscript{50} | \(n_I\) | \(I_{\text{max}}\) | \(n\) |
|--------------|-----------------------|--------|------------------|------|
| A288G        | 0.032 ± 0.008         | 1.8 ± 0.4 | 4.3 ± 0.2 | 4    |
| A288G/Y222C  | 0.1 ± 0.1\textsuperscript{a} | 3.5 ± 0.1\textsuperscript{b} | 6.8 ± 2.4 | 4    |
| A288G/I225W  | 2.4 ± 0.5\textsuperscript{c} | 2.5 ± 0.2 | 4.9 ± 0.3 | 5    |
| A288G/I229W  | 0.15 ± 0.02\textsuperscript{d} | 3.7 ± 0.2\textsuperscript{a} | 6.5 ± 0.7\textsuperscript{a} | 3    |
| A288G/Q226W  | 0.15 ± 0.02\textsuperscript{d} | 3.2 ± 0.4 | 5.8 ± 0.6\textsuperscript{b} | 3    |
| A288G/S229W  | 0.24 ± 0.02\textsuperscript{d} | 4.0 ± 0.4\textsuperscript{a} | 6.0 ± 0.7\textsuperscript{b} | 3    |
| A288G/P230W  | 5.6 ± 0.3\textsuperscript{a} | 2.3 ± 0.4 | 4.7 ± 0.8 | 4    |
| A288G/L233W  | Inhibition, see text  |        |                  |      |
| A288G/I234W  | No expression         | 10     |                  |      |
| A288G/Y279F  | 0.7 ± 0.2\textsuperscript{a} | 3.0 ± 0.8 | 8.6 ± 1.1\textsuperscript{b} | 6    |
| A288G/L291W  | Inhibition, see text  |        |                  |      |

\(p < 0.001\) by unpaired \(t\) test relative to WT GlyR values.
\(p < 0.05\) by unpaired \(t\) test relative to WT GlyR values.
\(p < 0.01\) by unpaired \(t\) test relative to WT GlyR values.

(30, 31), at nAChRs to define transmembrane helical structure and dynamics (32, 33) and at P\textsubscript{2}X receptors to investigate movements induced by ivermectin (34). Glycine dose-response relationships were quantitated for all these mutant GlyRs to establish whether the substitutions were well tolerated by the receptor. As cells transfected with the I234W mutant GlyR exhibited no response to glycine or ivermectin, this mutant was not investigated further. Averaged dose responses for M1 domain mutant GlyRs are shown in Fig. 3A (left panel) with parameters of best fit summarized in Table 2. Similarly, averaged dose responses for GlyRs incorporating mutations in the M2 or M3 domains are shown in Fig. 3B (left panel) with mean parameters of best fit summarized in Table 2. Mean glycine EC\textsubscript{50} values of all mutant GlyRs were within an order of magnitude of the WT GlyR value, although significant reductions in
GlyR. Whereas the P230W mutation produced an ivermectin EC\textsubscript{50} value 7-fold higher than the WT value, the P230W/A288G value was 175-fold greater than the value of 0.032 nM at the A288G mutant GlyR. Similarly, mutations of residues surrounding Pro\textsuperscript{230} on the same face of M1, I225W/A288G, Q226W/A288G, and I229W/A288G mutant GlyRs, exhibited EC\textsubscript{50} values 5–8-fold higher than A288G mutant GlyR alone, whereas the single mutants did not differ from WT values (Table 4). Thus, Pro\textsuperscript{230} emerges as a crucial ivermectin sensitivity determinant at the A288G mutant GlyR.

As discussed below, the above results are consistent with ivermectin binding at the M3–M1 intersubunit interface. As several other residues in this region have previously been implicated as binding sites for alcohols, anesthetics, and neurosteroids in GABA\textsubscript{\alpha}\textsubscript{3}Rs and GlyRs, we tested whether these residues may also include ivermectin-binding sites. As Ser\textsuperscript{267} (at the 15’ position of the M2 domain) in the α1 GlyR has been implicated as an alcohol-binding site (36, 37), we investigated the ivermectin sensitivity of the S267I mutant GlyR. As summarized in Table 1, this mutant GlyR exhibited an identical ivermectin sensitivity to the WT GlyR, although it did show a tendency toward higher I\textsubscript{max} and n\textsubscript{H} values. The potency with which ivermectin potentiated EC\textsubscript{10} glycine currents at the S267I mutant GlyR was also similar to WT (Table 3). Thus, Ser\textsuperscript{267} does not contribute to an ivermectin site. In the GABA\textsubscript{\alpha}\textsubscript{3}R, an α1 subunit M1 domain 12’ Thr residue (corresponding to Ile\textsuperscript{234} in the GlyR) has been proposed to contribute to an intersubunit neurosteroid site that is eliminated via the Thr-to-Ile mutation and only slightly altered by the Thr-to-Ser mutation (38). As the α1 GlyR contains an endogenous Ile at this position, we investigated the effect of the reverse I234S
As shown in Table 1, this mutation had no effect on ivermectin sensitivity. A GABA\(\alpha\)R etomidate-binding determinant at an \(\alpha\)-subunit Met residue corresponding to Leu\(^{233}\) in the \(\alpha\)I GlyR (27) can also be eliminated as a potential ivermectin sensitivity determinant on the grounds that the L233W mutation had no effect on ivermectin potency (Table 1). Finally, a GABA\(\alpha\)R neurosteroid-binding site at a \(\gamma\)2-subunit Tyr residue corresponding to Trp\(^{286}\) at the GlyR was shown to be eliminated by a Tyr-to-Phe substitution (38). However, the \(\alpha\)I GlyR W286F mutation produced only a moderate (2-fold) increase in the ivermectin \(EC_{50}\) value (Table 1). We also investigated the less conservative W286A mutation, but it did not express. Thus, we conclude that none of these residues are likely to contribute to an ivermectin-binding site.

**Evidence for Ivermectin-binding Site Spanning Adjacent Subunits**—To discriminate experimentally between intrasubunit and intersubunit locations of the ivermectin-binding site, we examined the ivermectin sensitivity of GlyRs formed from co-expression of P230W/A288G and A288F mutant subunits, which have low (5.6 \(\mu\)M) or no ivermectin sensitivity, respectively. Our rationale was that if the ivermectin-binding site is formed within a single subunit, then the resulting mixed receptors should be ivermectin-insensitive (i.e. \(EC_{50}\) > 5 \(\mu\)M) as each subunit is individually ivermectin-insensitive. Alternatively, if the resultant recombinant receptors are potently activated by ivermectin, then this site must be formed at those subunit interfaces that contain the two residues “permissive” for ivermectin sensitivity; (+) M3 288-Gly (from P230W/A288G mutant) and (−) M1 230-Pro (from A288F mutant). Assuming subunits recombine randomly to produce receptors with all possible stoichiometries, the number of putative intersubunit ivermectin sites (i.e. interfaces containing Gly\(^{288}\) on one face and Pro\(^{230}\) on the other) will range from 0 to 2 per receptor (17). As shown in the example in Fig. 5A, ivermectin did indeed potently activate currents in receptors formed by co-expression of P230W/A288G and A288F mutant subunits. These currents exhibited a mean ivermectin \(EC_{50}\) value of 0.7 ± 0.3 \(\mu\)M, an \(n_H\) of 1.5 ± 0.1,
and an $I_{\text{max}}$ of $3.1 \pm 1.0$ nA (all $n = 4$). This EC$_{50}$ shows higher sensitivity than the sensitivity of each subunit when expressed alone (Fig. 5B), suggesting that ivermectin sites are located at subunit interfaces and that potent receptor activation can be achieved with two bound ivermectin molecules. Our model below places these two residues directly opposite each other at the opening to a cavity at the intersubunit interface.

Selamectin Exhibits Reduced Agonist Efficacy at a1 GlyR and a3B GluClR—In an attempt to define the molecular interaction between ivermectin and the GlyR, we sought to identify moieties crucial for its potency and efficacy. We previously showed that the direct agonist EC$_{50}$ and $I_{\text{max}}$ values for doramectin, emamectin, eprinomectin, and moxidectin did not differ significantly from those for ivermectin at either the WT or A288G GlyRs (21). All these compounds share a common structure at the benzofuran moiety but vary in structure at other groups. As selamectin differs in structure from these compounds at the benzofuran moiety, we compared the effects of selamectin and ivermectin at the a1 GlyR. Selamectin contains an NOH group at the C05 position, whereas the other derivatives contain an OH group (abbreviated hereafter as C05-NOH and C05-OH, respectively). Selamectin, at concentrations up to 30 μM, activated no current at the WT GlyR. However, as shown in the example in Fig. 6A, it potently potentiated glycine currents. The averaged selamectin potentiating dose-response relationship, together with that for ivermectin reproduced from Fig. 1F, is shown in Fig. 6B. Selamectin potentiation exhibited a mean EC$_{50}$ of $3.6 \pm 0.9$ μM and an $n_{H_i}$ of $3.2 \pm 0.7$ (both $n = 5$). As both values did not differ significantly from those for ivermectin at the WT GlyR (Table 4), we tentatively conclude that selamectin binds with a similar potency to ivermectin but is unable to directly gate the receptor.

The A288G GlyR was directly activated by selamectin with a mean EC$_{50}$ value of $2.0 \pm 0.3$ μM, an $n_{H_i}$ value of $2.1 \pm 0.1$, and an $I_{\text{max}}$ of $2.6 \pm 0.2$ nA (all $n = 4$). As this selamectin EC$_{50}$ value was significantly higher than that of ivermectin at this mutant GlyR ($p < 0.001$), we concluded that the ivermectin C05-OH group was essential for high efficacy at highly ivermectin-sensitive Cys-loop receptors as well.

Molecular Modeling of a Putative Ivermectin-binding Site—To model the ivermectin-binding site, we carried out computational docking of ivermectin to our ELIC-based (Protein Data Bank code 2VL0) a1 GlyR model within a large box surrounding Ala$^{288}$. We found no significant differences in docking results in the WT relative to the A288G mutant GlyR (Fig. 7, A–C). As noted above, Ala$^{288}$ and Pro$^{280}$ (Fig. 7, shown in red and magenta, respectively) face each other across the subunit interface, either side of the opening to a cavity at the intersubunit interface. We hypothesized that the ivermectin-binding site may be within this cavity, but access of ivermectin to the cavity may require significant conformational change, not catered for in our model, even though we allowed flexible side chains for seven residues surrounding Ala$^{288}$. Consistent with this hypothesis of a cavity site, Thr$^{265}$ (from (+) side, shown in green) and Gln$^{266}$ (from (−) side, shown in blue) contribute to the lining of the cavity, and Trp substitutions of these residues caused an inhibitory effect of ivermectin and an increased ivermectin sensitivity, respectively. Other residues that, in the A288G background, showed reduced ivermectin sensitivity when substituted with Trp, Ile$^{225}$, Gln$^{226}$, and Ile$^{229}$ also surrounded the entrance to the cavity (shown in Fig. 7, A–C, light pink). The residues Leu$^{233}$ and Leu$^{291}$ (shown in yellow), that when substituted with Trp abolished activation by ivermectin but retained sensitivity to ivermectin in the form of increased desensitization, also face each other across the subunit interface one helical turn below Pro$^{280}$ and Ala$^{288}$, respectively.

Our binding site hypothesis independently corroborates the recently published α GluClR crystal structure with ivermectin bound (10). We then employed the same procedure as above to dock ivermectin onto a model of the α GlyR based on the α GluClR structure (Protein Data Bank code 3RIF). As shown in Fig. 7, D and E, this produced a binding orientation very similar to that seen in the crystal structure, suggesting that ivermectin binds in a similar pose to both receptors. This in turn suggests that the M1 and M3 domains in our original ELIC-based model were located too close to each other to allow ivermectin access to its binding site in the intersubunit cavity. Interestingly, we saw only slight differences in the orientation of ivermectin docked to WT and A288G GlyRs, colored gray and green, respectively, in Fig. 7, B and C, with only a slight shift to accommodate the extra bulk of the Ala$^{288}$ methyl group. Its predicted binding energies to the WT and A288G models were also similar.

Comparison of a1 GlyR with a GluClR Ivermectin Binding Interactions—Fig. 8 shows a sequence alignment of M1, M2, and M3 domains of the a1 GlyR and the crystallized C. elegans α GluClR. It also includes several other GluClRs to be considered below. Using α GluClR numbering, Leu$^{218}$, Ser$^{260}$, and Thr$^{285}$ were seen to form crucial H-bonds with ivermectin in the crystal structure (10). These residues (plus their homologues in other receptors) are colored blue in the alignment of Fig. 8, and the residues seen to form van der
Waals interactions with ivermectin are colored green. The residues identified in this study as crucial ivermectin determinants in the α1 GlyR (i.e. Pro230 and Ala288) are colored red in Fig. 8.

The H-bond with Leu218 is formed with the backbone carbonyl so its role in ivermectin binding is not readily tested by mutagenesis. Nevertheless, as the availability of the Leu218 carbonyl is due to helical disruption by the conserved M1 proline one helical turn lower, it is likely that this bond is conserved in other Cys-loop receptors.

As we had not probed the role of the M3 H-bonding residue (Thr264 in α GluClR and Leu292 in α1 GlyR) above, we investigated the effect of the L292T (α GlyR→α GluClR) mutation. If ivermectin binds identically to α1 GlyRs and α GluClRs, this mutation should introduce an H-bond that increases ivermectin affinity. Conversely, even if no H-bond is formed, this mutation may enhance ivermectin affinity by increasing the space available for ivermectin to bind in the TMD interface crevice.

The L292T mutant α1 GlyR was found to exhibit a mean ivermectin EC50 of 0.29 ± 0.01 μM (n = 4 cells), significantly lower than that of the WT GlyR (p < 0.05).

In the α GluClR structure, Ser262 (Ser152) forms an H-bond with the ivermectin C05-OH that was proposed to be essential for direct agonist activation of Cys-loop receptors (10). Although our docking suggested a similar H-bond exists in the GlyR, we showed above that mutation of the equivalent residue S267I, which eliminates any H-bonding propensity, had no effect on ivermectin sensitivity (Table 1), indicating this bond is either not functionally important or is not present in the α1 GlyR.
Glycine Receptor Ivermectin-binding Site

DISCUSSION

Functional Evidence for α1 GlyR Ivermectin-binding Site at the TMD Subunit Interface—The A288F substitution abolished α1 GlyR sensitivity to both the direct agonist and glycine potentiating effects of ivermectin. The P230W substitution in M1 decreased sensitivity to the direct ivermectin agonist effect by 7-fold in the WT GlyR and by 100-fold in the A288G mutant GlyR. The P230W mutation also decreased the glycine potentiating effects of ivermectin (Table 3). Molecular modeling places Ala288 and Pro230 residues from the neighboring subunits into close proximity at the TMD intersubunit interface, at either side of the entrance to an interface cavity (Fig. 7). Our experiments employing heteromeric mutant subunits (Fig. 5) provide independent experimental support for an ivermectin site at this interface. However, computational docking using our ELIC-based GlyR model did not reveal a convincing binding site for ivermectin that would be significantly disrupted by either the A288F or P230W mutations.

Selectivity of Ivermectin Analogues—The most useful technique for functionally characterizing molecular interactions is mutant cycle analysis. Unfortunately, as commercially available ivermectin analogues invariably differ from one another by more than one molecular group, mutant cycle analysis is not currently feasible for probing ivermectin-binding interactions. We have previously shown that emamectin, eprinomectin, moxidectin, and doramectin, which all vary from ivermectin at more than one molecular group, mutant cycle analysis is not directly testable by conventional mutagenesis, our data (Tables 1 and 3) and docking studies are consistent with this notion.

Finally, Hibbs and Gouaux (10) reported a network of van der Waals interactions between the ivermectin disaccharides and residues in the α GluClR M2–M3 loop that they thought might be important for allosteric interactions with the LBD. We have previously reported that moxidectin, which lacks both sugars, is equipotent with ivermectin at the α1 GlyR (21), arguing against a crucial role for these interactions in the GlyR.

Ivermectin Gating Determinants at the LBD-TMD Interface—If ivermectin interacts with the TMD of the α1 GlyR, why is its ability to open the channel affected by mutations at the LBD-TMD interface? The L142C, Y222C, and Y279F mutations each caused a large rightward shift in the ivermectin activation EC_{50} gies at WT and A288G GlyRs (Fig. 7). Thus, our model provides no clear explanation for the observed EC_{50} differences. We postulate that rather than affecting binding per se, the methyl group of Ala288 reduces access to the interface cavity and consequently reduces the on-rate and apparent affinity for ivermectin. The inhibition of access to the cavity would be greatly amplified in the A288F mutant, with the possible additional effect of direct steric inhibition of ivermectin binding.

Hibbs and Gouaux (10) identified an H-bond between Ser260 (Ser15^C1) and ivermectin C05-OH in the α GluClR that they proposed was crucial for both high ivermectin affinity and direct agonist efficacy. However, we showed this bond is not required for high affinity ivermectin activation of the α1 GlyR. Moreover, because the H. contortus α GluClR, the C. elegans α3B GluClR, and the H. contortus α3B GluClR all contain endogenous Ala residues at the corresponding 15^ position (Fig. 8) and are at least as ivermectin-sensitive as the α GluClR (40, 43), it is evident that this H-bond is not necessarily required for high ivermectin sensitivity or direct ivermectin activation in those receptors as well. If a slightly greater distance cutoff is allowed for defining H-bonds, then other potential H-bonds can be identified between ivermectin C05-OH and Gln219 in M1 and Asn264 in M2 of the α GluClR structure and equivalent residues Gln226 and Arg277 in our 3RIF-based GlyR model. Perhaps these H-bonds render the H-bond with Ser15^C1 functionally redundant.

Hibbs and Gouaux (10) also identified a crucial H-bond between the ivermectin spiroketal oxygen and an M3 Thr (Thr285 using α GluClR numbering). As the α1 GlyR contains a non-H-bonding leucine (Leu292) at the corresponding position (Fig. 8), it is evident this H-bond is not required for high ivermectin sensitivity at the WT or A288G α1 GlyRs. Moreover, as several other GluClRs contain an endogenous Ala at this position (Fig. 8), it is evident this H-bond is not required for high ivermectin sensitivity at some GluClRs as well. Nevertheless, we found that the α1 GlyR L292T mutation did increase ivermectin sensitivity, possibly consistent with a role for this H-bond.

The third H-bond, between ivermectin and the Leu218 backbone of the α GluClR, occurs because of the break in the helix due to the highly conserved M1 Pro. As the α1 GlyR contains this Pro and a conserved residue (Ile225^C1) at the Leu position, we infer the same H-bond is likely to exist in the α1 GlyR. Although not directly testable by conventional mutagenesis, our data (Tables 1 and 3) and docking studies are consistent with this notion.

Ivermectin Gating Determinants at the LBD-TMD Interface—If ivermectin interacts with the TMD of the α1 GlyR, why is its ability to open the channel affected by mutations at the LBD-TMD interface? The L142C, Y222C, and Y279F mutations each caused a large rightward shift in the ivermectin activation EC_{50}
value, although ivermectin potentiation of glycine currents showed a similar sensitivity as seen in the WT GlyR. The same mutations also produced large rightward shifts in glycine activation $EC_{50}$ values (Table 2). Similar effects on ivermectin and glycine sensitivities were observed when the above three mutations were investigated on the background of the A288G mutation. Together, these results strongly suggest that the L142C, Y222C, and Y279F mutations do not disrupt an ivermectin-binding site but rather reduce ivermectin gating efficacy. This is consistent with the known roles of the conserved Cys-loop, the pre-M1 domain, and the M2–M3 domain in gating the GlyR (44) and suggests that ivermectin activation involves a global conformational change that propagates to the LBD rather than simply a localized TMD conformational change. We therefore speculate that other ivermectin sensitivity determinants that have previously been identified in the TMD and interfacial domains of GluClRs (7, 9, 40) also disrupt the gating rather than the binding mechanisms of ivermectin.

**Allosteric Effects of TMD Mutations Near the Ivermectin Site**—
The P230W mutation decreased both glycine sensitivity and ivermectin sensitivity. It appears the dominant effect of this mutation is to rearrange the M1 helix so as to abolish the H-bond between ivermectin and the Ile225 backbone. It is interesting that the Q226W, P230W, and I234W mutant GlyRs all showed decreased glycine sensitivities (with no function at all for I234W) compared with the I225W, I229W, and L233W mutant GlyRs, suggesting that the effects of P230W are not specific to ivermectin. However, the decrease in glycine sensitivity caused by the Trp substitution at Gln226, Pro230, and Ile234 might simply reflect their apposition with M3 of the adjacent subunit, whereas the Trp substitutions at Ile225, Ile229, and Leu233 are less disruptive because their side chains are directed toward the surrounding lipids (45). However, it is possible that Pro230 mutations might also affect ivermectin gating efficacy for two main reasons. The first is that Pro230 substitutions will remove a central kink in the M1 $\alpha$-helix that will alter interactions between the helix and neighboring domains throughout its length. This could have wide ranging effects on allosteric interactions involving this domain. The second related reason is that the corresponding Pro-to-Ala mutation in the $\beta2$ subunit of the $\alpha1$2$\beta2$2 GABA$_A$R affects both GABA sensitivity and barbiturate potentiation (46), consistent with a disruption of the linkage between the GABA-binding site and the channel gate.

The threshold concentrations at which ivermectin begins to enhance desensitization at L233W and L291W mutant GlyRs are 1–10 $\mu$M, not remarkably different from WT ivermectin $EC_{50}$ values. The glycine sensitivities of both mutant receptors are also significantly higher than WT GlyR values (Table 2). The nonconservative L233Q and L291Q substitutions had no effect on the mode of action of ivermectin, implying that the large hydrophobic Trp side chain is required for this effect, potentially blocking the conformational change normally favored by ivermectin. We therefore conclude that ivermectin binds with normal affinity to the L233W and L291W mutant GlyRs but promotes a desensitized state, rather than an open state. From our results, it is unclear if this effect is due to an altered ivermectin binding conformation imposed by the Trp

**Conclusion**—Our docking simulations predict that ivermectin binds to the $\alpha1$ GlyR in a similar pose as observed in the $\alpha$ GluClR crystal structure. Ivermectin binding to the $\alpha$ GluClR was shown to be mediated by H-bonds with Leu1218, Ser260, and Thr285 and a network of van der Waals interactions (10). We showed that H-bonds with residues equivalent to Ser260 and Thr285 are not required for high ivermectin sensitivity at either the $\alpha$ GlyR or three other GluClRs. As the H-bond with the Leu218 or equivalent residues (e.g. GlyR Ile225) is via the carbonyl backbone, its role cannot be readily tested and is likely to be conserved along with the conserved Pro that exposes this carbonyl. We also show that van der Waals interactions between the ivermectin sugars and $\alpha$ GlyR M2–M3 loop residues are not important for high ivermectin affinity or efficacy.

The $\alpha1$ GlyR A288F mutation eliminated the direct activation and glycine-potentiating effects of ivermectin, presumably by blocking access to the cavity. The effect of this mutation contrasted with those of several mutations in the conserved Cys-loop, pre-M1 domain, and M2–M3 loop that disrupted only the direct activation but not the glycine-enhancing effects of ivermectin. We conclude these later residues disrupted ivermectin gating efficacy, consistent with the known role of this domain in mediating glycine agonist transduction.

In summary, our results indicate that the ivermectin-binding mechanisms as revealed by the $\alpha$ GluClR structure cannot be generalized to other Cys-loop receptors with comparable ivermectin sensitivities. Understanding these mechanisms will be crucial for designing new drugs as anthelmintics and as therapies for a wide range of human neurological disorders.

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Glycine Receptor Ivermectin-binding Site

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