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Ivermectin, an Unconventional Agonist of the Glycine Receptor Chloride Channel*

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The effects of the antihelmintic, ivermectin, were investigated in recombinantly expressed human α_1 homomeric and $\alpha_1\beta$ heteromeric glycine receptors (GlyRs). At low (0.03 μM) concentrations ivermectin potentiated the response to sub-saturating glycine concentrations, and at higher (≥0.03 μM) concentrations it irreversibly activated both α_1 homomeric and $\alpha_1\beta$ heteromeric GlyRs. Relative to glycine-gated currents, ivermectin-gated currents exhibited a dramatically reduced sensitivity to inhibition by strychnine, picrotoxin, and zinc. The insensitivity to strychnine could not be explained by ivermectin preventing the access of strychnine to its binding site. Furthermore, the elimination of a known glycine- and strychnine-binding site by site-directed mutagenesis had little effect on ivermectin sensitivity, demonstrating that the ivermectin- and glycine-binding sites were not identical. Ivermectin strongly and irreversibly activated a fast-desensitizing mutant GlyR after it had been completely desensitized by a saturating concentration of glycine. Finally, a mutation known to impair dramatically the glycine signal transduction mechanism had little effect on the apparent affinity or efficacy of ivermectin. Together, these findings indicate that ivermectin activates the GlyR by a novel mechanism.

Ivermectin (22,23-dihydroavermectin B_{1a}) is macrocyclic lactone widely used as an antiparasitic agent in domestic animals and is considered the drug of choice for lymphatic filariasis and river blindness (onchocerciasis) in humans (1–3). The target of its antiparasitic action is believed to be an ivermectin-sensitive glutamate-gated Cl^- channel receptor (GluClR)[1] that exists in a number of invertebrate phyla (4–7). Ivermectin also acts as an anticonvulsant in a variety of vertebrate seizure models (8–10). However, GluClRs have not been demonstrated to exist in vertebrates, and the anticonvulsant actions of ivermectin in a mouse seizure model has recently been shown to be mediated by GABA type A receptors ($GABA_A$Rs) (10).

The GluClR and the $GABA_A$R belong to the ligand-gated ion channel superfamily, which also includes the nicotinic acetylcholine receptor cation channel (nAchR), the serotonin type 3 receptor cation channel, and the glycine receptor chloride channel (GlyR) (4, 11–14). The mechanisms of action of ivermectin and its analogues have been investigated in several members of this family. For example, ivermectin irreversibly activates the GluClR over a concentration range from 0.1 to 1 μM, although at lower concentrations (<0.01 μM) it induces potentiation of glutamate-gated currents (4, 5). The effects of ivermectin on the $GABA_A$R also include potentiation of GABA-gated currents (10, 15, 16) as well as direct, reversible receptor activation (10, 17, 18), although both effects have not always been observed in some preparations. In addition, ivermectin has been shown to potentiate acetylcholine-mediated responses in the recombinantly expressed α_7 nAChR (19). Finally, ivermectin has long been known to displace [^{3}H]strychnine in radiolabeled binding studies (20), implying that it may exert some effect on the GlyR. Indeed, ivermectin has recently been demonstrated to act as a use-dependent inhibitor of a GlyR that is endogenously expressed in primary cultured rat cortical neurons (10).

The effects of ivermectin on a variety of members of the ligand-gated ion channel superfamily prompted us to test its actions on recombinantly expressed human α_1 homomeric and $\alpha_1\beta$ heteromeric GlyRs. We find that ivermectin acts as an allosteric potentiator of glycine-gated currents at low (0.03 μM) concentrations and as a potent, irreversible agonist at higher concentrations. In addition, since ivermectin affinity is not affected by mutations to a well characterized glycine binding domain, and the pharmacology of ivermectin-gated currents is different to that of glycine-gated currents, it appears that ivermectin acts via a novel mechanism to activate the GlyR.

EXPERIMENTAL PROCEDURES

Mutagenesis and Expression of GlyR cDNAs—The human GlyR α_1 and β subunit cDNAs were subcloned into the pCIS2 and pIRES2-EGFP plasmid vectors (CLONTECH, Palo Alto, CA), respectively. Because GlyR α subunits can efficiently assemble into functional GlyRs as either α homomers or α/β heteromers, green fluorescent protein expression was used as an indicator of GlyR β subunit expression. Site-directed mutagenesis was performed using the QuickChange mutagenesis kit (Stratagene, La Jolla, CA), and the successful incorporation of mutations was confirmed by sequencing the clones. Adenovirus-transformed human embryonic kidney 293 cells (ATCC CRL 1573) were passaged in minimum essential medium supplemented with 2 mM glutamate, 10% fetal calf serum, and the antibiotics penicillin 50 IU/ml and streptomycin 50 μg/ml (Life Technologies, Inc.). Cells were transfected using a calcium phosphate precipitation protocol (21). When cotransfecting the GlyR α and β subunits, their respective cDNAs were combined in a ratio of 1:10 (22). After exposure to transfection solution for 24 h, cells were washed twice using the culture medium and used for recording over the following 24–72 h.

Electrophysiology—The cells were observed using a fluorescent microscope, and currents were measured using the whole cell patch clamp configuration. Cells were perfused by a control solution that contained (in mM) 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 10 glucose, with the pH adjusted to 7.4 with NaOH. Patch pipettes were fabricated from borosilicate hematocrit tubing (Vitrex, Modulohm, Denmark) and heat-polished. Pipettes had a tip resistance of 1.5–3 MΩ when filled with the standard pipette solution which contained (in mM) 145 CsCl, 2 $CaCl_2$, 2

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[1] The abbreviations used are: GluClR, glutamate-gated Cl^- channel receptor; ANOVA, analysis of variance; GABA, γ-aminobutyric acid; $GABA_A$Rs, GABA type A receptors; nAchR, nicotinic acetylcholine receptor; GlyRs, glycine receptors; WT, wild-type.

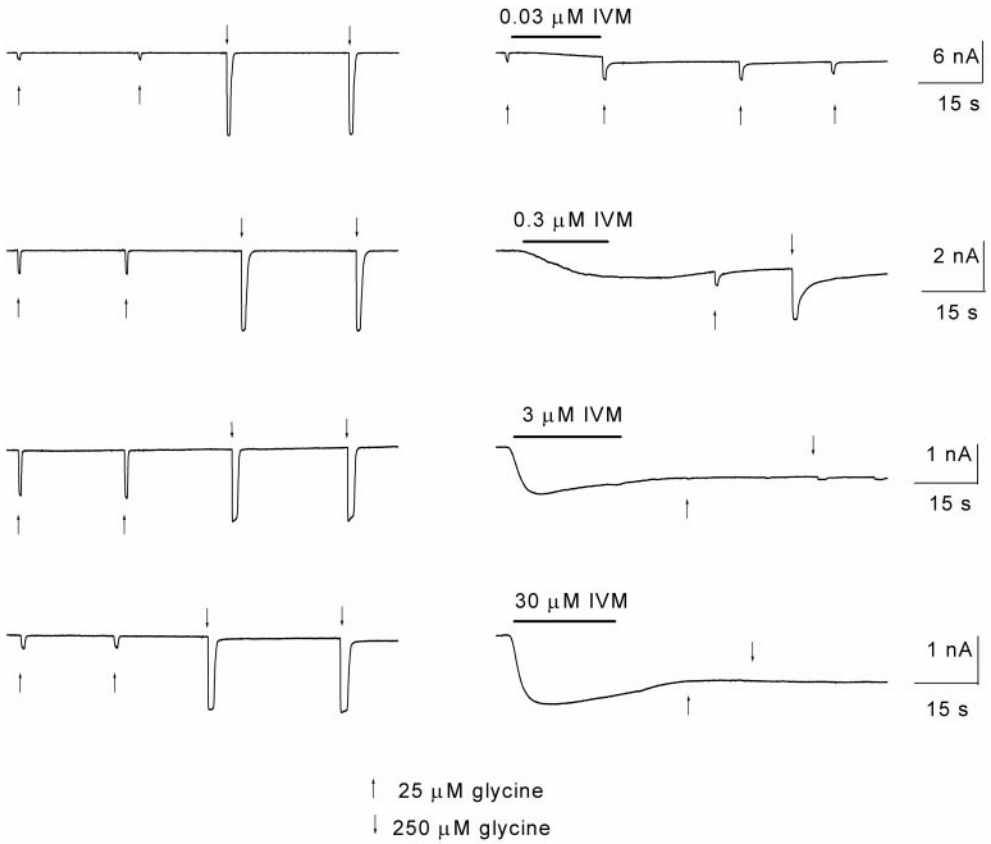

FIG. 1. **Ivermectin potentiation and activation of the GlyR.** In this and all subsequent figures, all displayed traces were recorded from cells expressing WT or mutated α_1 homomeric GlyRs using whole cell recording. In this figure, traces in different rows were obtained from different cells, although both traces on a single row were from the same cell. Each cell was treated first with two applications of a half-saturating (25 μM) glycine concentration, followed by two applications of a saturating (250 μM) glycine concentrations (*left panels*). Following this, ivermectin was applied at the indicated concentration, and glycine responses were recorded again (*right panels*).

$MgCl_2$, 10 HEPES, 10 EGTA, with the pH adjusted to 7.4 with NaOH. After establishment of the whole cell configuration, cells were voltage-clamped at −40 mV, and membrane currents were recorded using an Axopatch 1D amplifier and pCLAMP7 software (Axon Instruments, Foster City, CA). The cells were perfused by a parallel array of microtubular barrels through with solutions were gravity-induced. The amplifier series resistance compensation was used to compensate for at least 50% of the series resistance error. Experiments were conducted at room temperature (18–22 °C).

Ivermectin (Sigma) was stored frozen as a 10 mM stock solution in dimethyl sulfoxide for up to 2 weeks. When dissolved into the perfusion solution, the final concentration of dimethyl sulfoxide was no more than 0.3%. Strychnine, picrotoxin, and zinc were also prepared from frozen stocks at concentrations of 10 mM (in water), 100 mM (in dimethyl sulfoxide), and 100 mM (in water), respectively.

The effects of ivermectin were tested with the following procedure. First, the glycine dose-response was measured by applying increasing concentrations of glycine at 30-s intervals. Then two brief applications of glycine at the half-saturating concentration (EC_{50}) were followed by two brief applications at a saturating concentration ($10 \times EC_{50}$), all at 30-s intervals. Provided the current amplitude remained constant, the averaged current amplitudes were used as the control. Following this, ivermectin was applied until a steady-state response was attained (usually <1 min). Because ivermectin induced irreversible activation, only a single ivermectin treatment was obtained per cell, and the coverslip was discarded after each recording.

Data Analysis—Glycine dose responses were measured by applying a series of glycine concentrations to each cell. Glycine dose-response parameters were quantitated by fitting the Hill equation to individual dose responses by a nonlinear curve fitting routine (Origin 4.0, Northampton, MA). The EC_{50} and Hill coefficient (n_H) values thus obtained were then averaged as means ± S.E. The irreversible nature of ivermectin activation meant that only one concentration could be applied on one cell. In this case, current amplitude was normalized to the saturating ($10 \times EC_{50}$) glycine-induced current in the same cell and then averaged with the data recorded from other cells at the same concentration. The pooled results recorded at different concentrations from different cells were then fitted with the Hill equation to obtain the $I_{max}/I_{max(Gly)}$, EC_{50}, and Hill coefficient (n_H) values. The n_H values obtained in such a manner must be interpreted with caution as curve fits to averaged dose responses typically underestimate its true value. Because of this uncertainty, the present study avoids drawing inferences from n_H data. Where possible, statistical significance was determined by one-way ANOVA, with $p < 0.05$ representing significance. However, because only a single EC_{50} value was obtained for the pooled ivermectin dose responses, a simple one-way ANOVA could not always be performed. In such cases the statistical significance of differences in ivermectin responses between wild-type and mutant GlyRs was determined with a general linear model of two-way analysis of variance (ANOVA) (Minitab 13.20, State College, PA), with the two factors being ivermectin concentration and phenotype, with $p < 0.01$ representing significance. The p values thus obtained using this analysis are shown in Table II.

RESULTS

Ivermectin Activation of the GlyR—Examples of the effects of ivermectin on wild-type (WT) α_1 homomeric GlyRs are shown in Fig. 1. It can be seen that ivermectin, at concentrations of 0.3 μM or greater, induced irreversible channel activation. Such effects were not reversed by a 10-min wash in ivermectin-free control solution. Because of its irreversible effect, only one ivermectin application could be recorded from each cell. Hence, the traces in Fig. 1 corresponding to different ivermectin concentrations were recorded from different cells, although both traces in any given row were recorded from the same cell. It is notable that the ivermectin-induced activation was much slower than that induced by glycine, particularly at the lower ivermectin concentrations. Although a 0.03 μM application of ivermectin activated little current, it did result in a strong

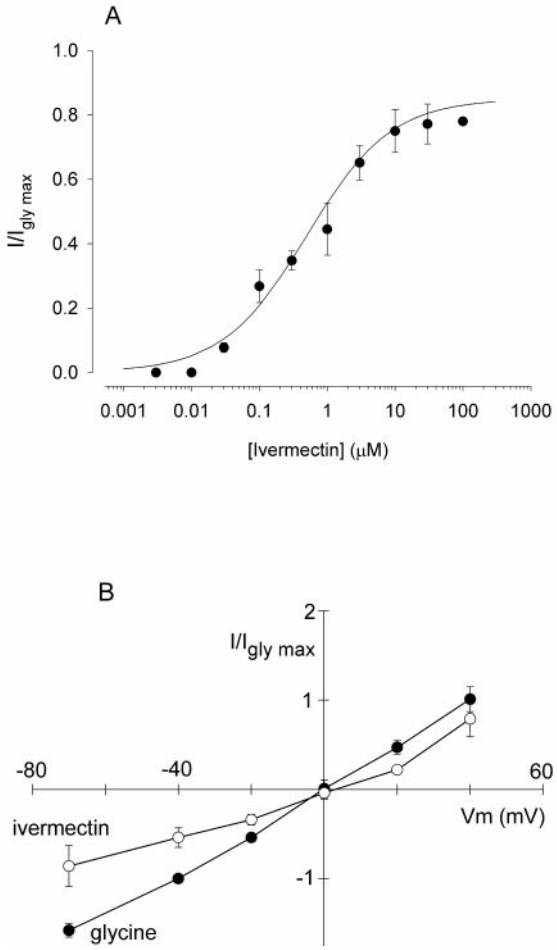

FIG. 2. **Properties of ivermectin-gated currents.** *A,* the ivermectin dose-response curve was compiled as described in the text. The *curve* was fitted with an EC_{50} of 0.39 μM and an n_H of 0.59. *B,* current-voltage relationship curves for glycine- and ivermectin-gated currents were determined as described in the text. Current amplitude was normalized to the saturating concentration (250 μM) glycine-induced current recorded at −40 mV. The reversal potential was −0.2 mV for glycine and 3.7 mV for ivermectin.

potentiation of the current activated by a sub-saturating glycine concentration (Fig. 1, *top panel*). This effect is considered further below. Ivermectin was a partial agonist of the GlyR, with a saturating (30 μM) concentration activating 77 ± 6% (n = 6) of the current activated by a saturating (250 μM) concentration of glycine. When ivermectin was applied at a saturating concentration, glycine activated no additional current (Fig. 1, *bottom panel*), indicating that ivermectin and glycine compete for the activation of the same population of GlyRs.

The ivermectin dose-response relationship was constructed by normalizing the magnitude of the current activated by a known concentration of ivermectin to the magnitude of the current activated by a saturating concentration (10 × EC_{50}) of glycine in the same cell. Thus, a single point on a dose-response curve was obtained from each cell. By measuring the relative magnitudes of ivermectin-gated currents at a single concentration in many cells, a pooled ivermectin dose response was compiled as shown in Fig. 2*A*. Each point in this figure was averaged from at least three different cells. The curve, which was fit to all points, had an EC_{50} of 0.39 μM and an n_H of 0.59. The ivermectin EC_{50} and n_H values for all other GlyR constructs investigated in this study were calculated in the same way, and all results are summarized in Table I. The respective glycine EC_{50} and n_H values for the same GlyR constructs were calculated from entire dose responses measured in individual cells, and these results are also summarized in Table I.

GlyRs *in vivo* are considered to exist as heteromers comprising α and β subunits in the ratio 3:2 (23). Since the α_1/β heteromeric GlyR differs pharmacologically from the α_1 homomeric GlyR (22, 24), it was possible that the heteromers may respond differently to ivermectin. However, the presence of the β subunit had no significant effect on ivermectin sensitivity (Table I). Hence, all experiments described in the remainder of this paper were performed on α_1 homomeric GlyRs.

We then investigated the ivermectin current-voltage (I-V) relationship of the WT GlyR according to the following procedure. First, a saturating concentration (0.25 mM) of glycine was applied at −40 mV, followed by a repeated glycine application and a 3 μM (saturating) ivermectin application at a specific holding potential of −70, −40, −20, 0, 20, or 40 mV. The glycine- and ivermectin-induced currents measured at the specific holding potential were normalized to the initial glycine current recorded at −40 mV to generate the I-V curve displayed in Fig. 2*B*. This figure demonstrates that, in contrast to the linear I-V relationship that characterized glycine-gated currents, the ivermectin-gated currents displayed weak outward rectification. Differences in rectification between ivermectin- and glutamate-gated conductances have also been observed in the GluClR (4). The reversal potentials for currents activated by both glycine (−0.2 mV) and ivermectin (+3.7 mV) approximated to the equilibrium potential (0 mV) for chloride ions.

Ivermectin Potentiation of Glycine-gated Currents—As shown in Fig. 1 (*top panel*), low concentrations (0.03 μM) of ivermectin potentiated glycine-gated currents. Both the glycine and ivermectin dependence of this effect were investigated. As shown in the example in Fig. 3*A*, 0.03 μM ivermectin dramatically increased the magnitude of current activated by a 10 μM (EC_5) concentration of glycine (*upper panel*) but appeared to slightly diminish the amplitude of currents activated by a 250 μM (saturating) glycine concentration (*lower panel*). The magnitude of the potentiation induced by 0.03 μM ivermectin was measured at the following glycine concentrations (with approximate EC values in parentheses): 10 (EC_5), 25 (EC_{50}), 30 (EC_{60}), 100 (EC_{95}), and 250 μM (EC_{100}). The mean percentage change in glycine-gated current at each concentration is summarized in Fig. 3*B*. Since ivermectin had no significant effect at the EC_{60} glycine concentration, it is not possible to conclude that it acted by a simple enhancement of the apparent glycine affinity. The results suggest instead that ivermectin selectively potentiated currents activated by the lowest glycine concentrations, without dramatically changing the glycine EC_{50} value. The significant reduction in current magnitude at 250 μM glycine is expected since ivermectin is a partial agonist.

The range of ivermectin concentrations that induced potentiation of glycine currents was very narrow. In fact, 0.01 μM ivermectin induced no significant potentiation of glycine responses nor did it induce significant direct current activation (n = 4 cells). On the other hand, 0.1 μM ivermectin directly activated 27 ± 5% (n = 4 cells) of the saturating glycine-gated current but also induced no significant potentiation of glycine-gated currents (n = 4 cells). At a concentration of 0.03 μM, ivermectin exhibited a dual effect as a potentiator and an activator inducing 7.8 ± 1.5% (n = 4 cells) of the saturating glycine-gated current.

Pharmacology of Ivermectin-gated Currents—The pharmacological profile of ivermectin-gated currents was investigated by measuring the inhibitory potencies of strychnine, picrotoxin, and zinc. Strychnine is considered to act as a classical competitive antagonist of glycine with an affinity in the nanomolar

TABLE I

Summary of glycine and ivermectin effects on WT and mutant GlyRs

All mutations were incorporated into α homomeric GlyRs.

GlyR	Glycine			Ivermectin				
	EC_{50} (-fold change)	n_H	n	EC_{50} (-fold change)	n_H	I_{max}/I_{max}(Gly)	p	n^a
		mM			*μM*			
WT/α	0.026 ± 0.009 (1)	3.4 ± 0.31	4	0.39 (1)	0.59	0.77 ± 0.06[b]		42.6
WT/αβ	0.024 ± 0.005 (0.92)	3.0 ± 0.73	4	0.29 (0.74)	0.95	0.68 ± 0.04[b]	0.71	31.3
F159Y	0.012 ± 0.002 (0.46)	2.5 ± 0.24	4	0.56 (1.4)	1.26	0.83 ± 0.06[b]	0.44	18.3
Y161F	0.016 ± 0.006 (0.62)	1.7 ± 0.43	4	2.7 (6.9)	1.6	0.71 ± 0.08[b]	0.001	18.3
Y202F	5.5 ± 2.4 (212)	1.3 ± 0.17	6	1.9 (4.9)	0.79	0.81 ± 0.09[b]	0.003	27.3
T204A	6.4 ± 2.9 (246)	2.5 ± 0.80	4	2.6 (6.7)	3.6	0.83 ± 0.09[b]	0.09	18.3
I244A	0.78 ± 0.23 (30)	1.5 ± 0.26	4	0.32 (0.82)	0.82	1.7 ± 0.2[b]	<0.001	18.3
R271Q	7.1 ± 0.19 (273)	1.7 ± 0.20	5	7.9 (20)	3.2	4.7 ± 1.2[b]	0.009	17.4

[a] The first n value indicates the number of cells used to calculate the EC_{50} and the second indicates the number used to calculate $I_{max}/I_{max(Gly)}$.
[b] Significant differences between ivermectin- and glycine-gated currents using one-way ANOVA ($p < 0.05$) are shown. The p values for ivermectin were determined as outlined under "Experimental Procedures."

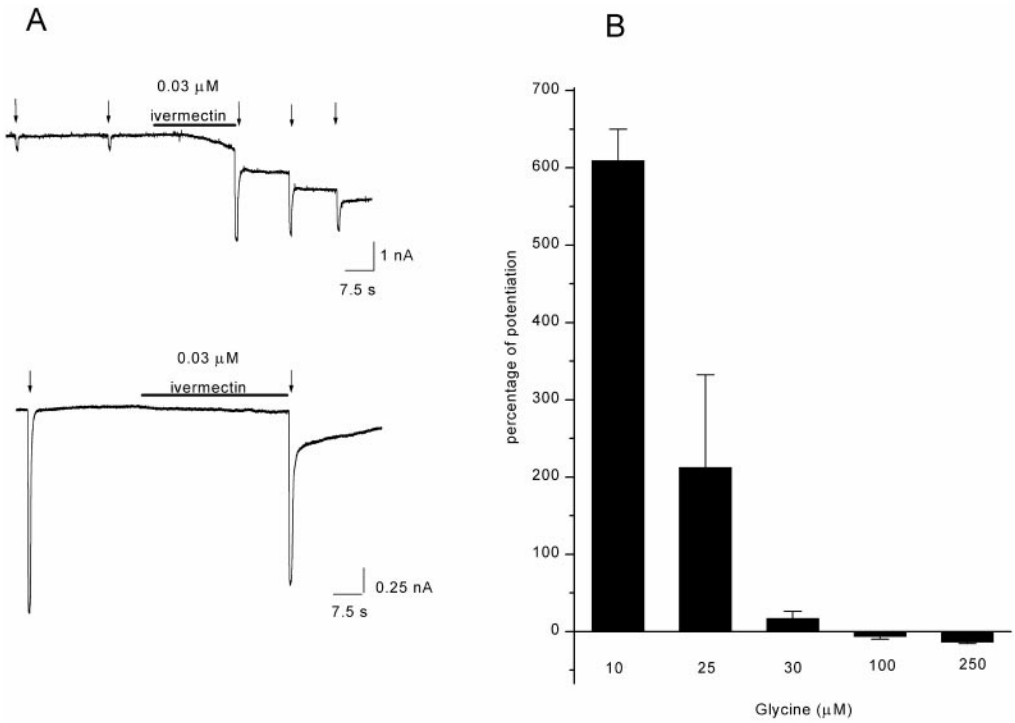

FIG. 3. **Ivermectin potentiation of submaximal glycine-gated currents.** *A,* pre-applied 0.03 μM ivermectin potentiated the currents activated by a 10 μM (EC_5) glycine concentration (*upper trace*) and slightly reduced the currents activated by a 250 μM (saturating) glycine-induced current (*lower trace*). Both traces were from different cells. *B,* glycine concentration dependence of potentiation by 0.03 μM ivermectin. All values were averaged from at least three cells.

range (25–27). As shown in Fig. 4*A* (*left panel*), 10 μM strychnine strongly inhibited the current activated by a saturating (250 μM) concentration of glycine. However, 10 μM strychnine had no significant inhibitory effect on the current activated by a saturating (3 μM) concentration of ivermectin. The effect of strychnine was also investigated at the lower ivermectin concentration of 0.3 μM, where it was found to induce significant inhibition. The effect of 10 μM strychnine on glycine- and ivermectin-gated currents are summarized in Table II. The results indicate that ivermectin-gated currents have a dramatically reduced sensitivity to strychnine.

Since ivermectin is an irreversible agonist, the reduction in strychnine efficacy could have resulted from the bound ivermectin sterically hindering strychnine from accessing its binding site. To investigate this possibility, we applied strychnine first and sought to determine whether pre-bound strychnine could inhibit the subsequent rate of activation of ivermectin-induced currents. The time constant of strychnine unbinding was estimated as indicated in Fig. 4*B*, *left panel*. Following removal of strychnine, the saturating current magnitude was regularly monitored by brief applications of 250 μM glycine. Fitting first-order exponential curves to the current peaks enabled us to estimate a mean unbinding time constant for 10 μM strychnine of 5.2 ± 0.26 s ($n = 5$) and for 100 μM strychnine of 22.1 ± 1.4 s ($n = 5$). The mean time constant of current activation by 3 μM ivermectin was 4.4 ± 1.0 s ($n = 6$). This value was not significantly different to the unbinding time constant of 10 μM strychnine (one-way ANOVA, $p > 0.05$) but was significantly faster than the unbinding time constant of 100 μM strychnine ($p < 0.05$). Therefore, if strychnine and ivermectin are competing for a common or overlapping binding site, the dissociation rate of 100 μM strychnine should be the rate-limiting step in the activation of ivermectin-gated currents. An example of an experiment designed to investigate this possibility is shown in Fig. 4*B*, *right panel.* When 3 μM ivermectin was applied immediately after a long (30 s) application of 100 μM strychnine, the mean current activation time constant was 2.9 ± 0.72 s ($n = 3$). This value was not signifi

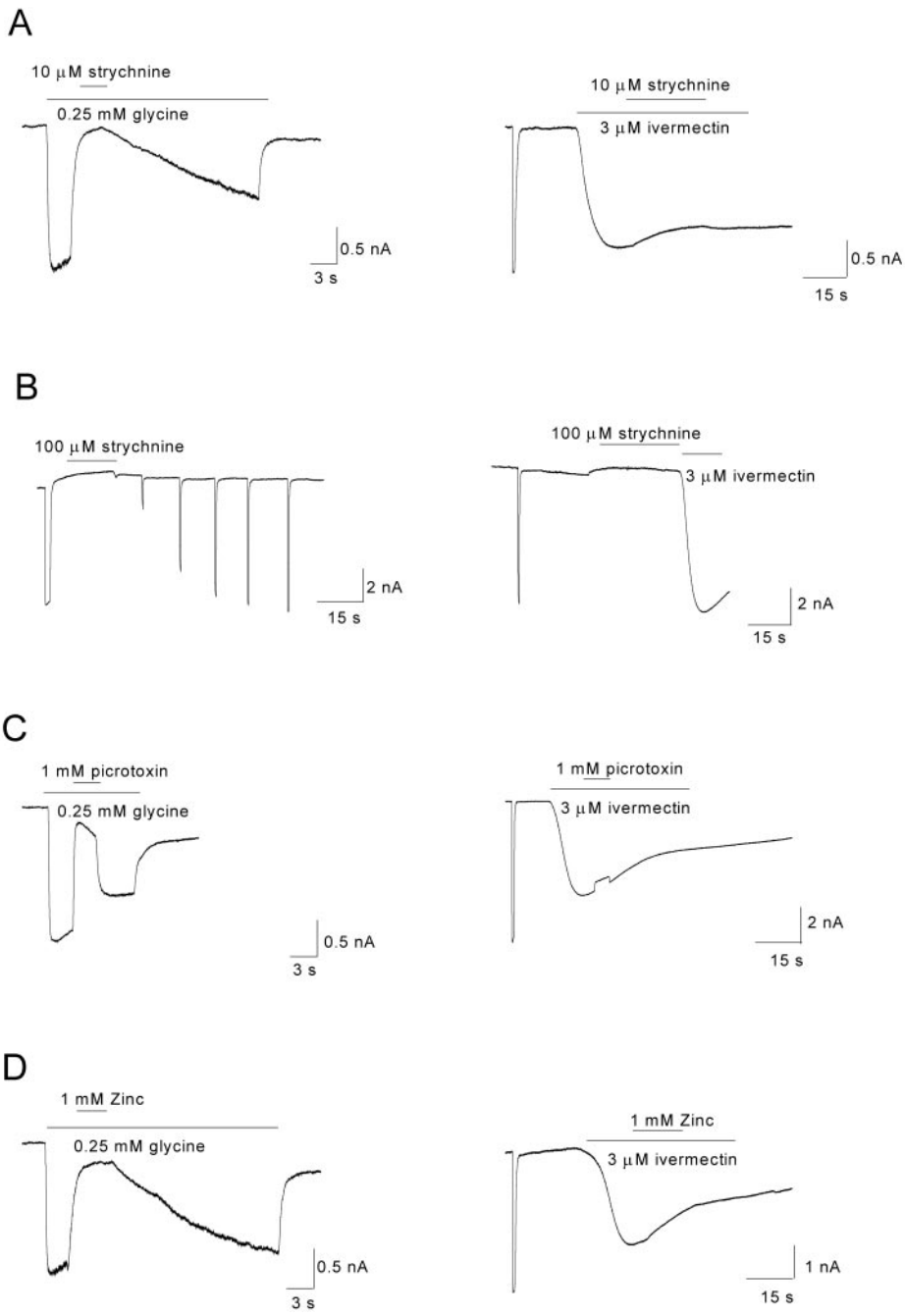

FIG. 4. **Pharmacology of ivermectin-gated currents.** *A,* a 10 μM concentration of strychnine strongly inhibits currents activated by a saturating (250 μM) concentration of glycine (*left panel*) but has little effect on the current activated by a saturating (3 μM) concentration of ivermectin (*right panel*). *B,* the time course of recovery from an application of 100 μM strychnine is slow and can be quantitated by regularly monitoring current with short applications of 250 μM glycine (*left panel*). However, a pre-application of 100 μM strychnine has little apparent effect on the rate of activation of ivermectin-mediated currents in the same cell (*right panel*). *C,* a 1 mM concentration of picrotoxin strongly inhibits currents activated by a saturating (250 μM) concentration of glycine (*left panel*) but has little effect on the current activated by a saturating (3 μM) concentration of ivermectin (*right panel*). *D,* similarly, 1 mM zinc strongly inhibits currents activated by a saturating (250 μM) concentration of glycine (*left panel*) but has little effect on the current activated by a saturating (3 μM) concentration of ivermectin (*right panel*).

cantly different to the activation time constant in the absence of strychnine (one-way ANOVA, $p > 0.05$), indicating that pre-bound strychnine has no effect on the activation rate of ivermectin-gated currents. Therefore, in contrast to its effect on glycine-gated currents, strychnine is a relatively weak antagonist of ivermectin-gated currents.

Although the mechanism of action of picrotoxin on the GlyR has not been unequivocally resolved, evidence to date indicates that it acts as an allosteric inhibitor (23, 28). As shown in Fig. 4*C* (*left panel*), 1 mM picrotoxin strongly inhibited currents activated by 250 μM glycine. However, in the same cell it was apparent that the same picrotoxin concentration had little effect on currents activated by 3 μM ivermectin (Fig. 4*C*, *right panel*). The inhibitory effects of picrotoxin on both glycine- and ivermectin-gated currents are summarized in Table II. The results indicate that picrotoxin is a comparatively poor antagonist of ivermectin-activated currents.

Zinc acts as an allosteric potentiator of glycine-gated currents at low (<3 μM) concentrations and as a competitive antagonist at higher concentrations (29, 30). As shown in Fig. 4*D*, 1 mM zinc strongly inhibited glycine-gated currents but had little effect on ivermectin-gated currents. The averaged results displayed in Table II confirm that zinc antagonism of ivermectin-gated currents is also very weak. Taken together, these results indicate that ivermectin-gated currents are pharmacologically distinct from glycine-gated currents.

Effects of Glycine-binding Site Mutations on Ivermectin-gated Currents—Previous studies have revealed that two regions in the GlyR α_1 subunit external N-terminal domain, Phe^{159}–Gly^{160}–Tyr^{161} and Lys^{200}–Tyr^{202}–Thr^{204}, are major determinants of the glycine- and strychnine-binding sites (26, 27, 31–33). Accordingly, the effects of ivermectin were investigated on the F159Y, Y161F, Y202F, and T202A mutant GlyRs. Since ivermectin remained an irreversible agonist of all these mutant GlyRs, the ivermectin dose responses were measured as described in Fig. 2*A*. The glycine dose responses for each mutant

TABLE II
Percentage inhibition by picrotoxin, strychnine, and zinc of glycine- and ivermectin-induced currents in the WT GlyR

Agonist	10 μM strychnine	1 mM picrotoxin	0.3 mM picrotoxin	1 mM Zn^{2+}
0.25 mM glycine	90 ± 4.7% (3)	96 ± 2.2% (3)		84 ± 5.1% (3)
0.3 μM ivermectin	40 ± 4% (4)[a]	34 ± 6% (5)[a]	9.3 ± 2.4% (3)[a]	14 ± 7.8% (3)[a]
3 μM ivermectin	0% (3)[a]	13 ± 8% (3)[a]		0% (3)[a]

[a] Significant differences with respect to WT GlyR values using one-way ANOVA ($p < 0.05$) are shown, and n values are given in parentheses.

GlyR were also measured, and all results are summarized in Table I. Consistent with previous studies (26, 27, 31–33), the glycine sensitivity was dramatically reduced (>200-fold) by the Y202F and T204A mutations, although the sensitivity was modestly increased by the F159Y and Y161F mutations. However, the ivermectin sensitivity was only weakly affected (1.4–6.9-fold) by any of these mutations (Table I). In addition, 0.03 μM ivermectin also potentiated half-saturating concentration glycine-induced current in each of the 4 mutant GlyRs (not shown). Thus, ivermectin does not act by binding to the glycine-binding site formed by Tyr^{202} and Thr^{204}.

Effect of a Startle Disease Mutation on Ivermectin Efficacy—R271Q is a heritable mutation in the human GlyR α_1 subunit that underlies familial startle disease (34). This mutation has previously been shown to induce a large reduction in both glycine sensitivity and single channel conductance (35, 36). In addition, this mutation also converts the glycinergic agonists, β-alanine and taurine, from agonists into competitive glycine antagonists (37, 38). The effects of this mutation are most likely to be mediated by a disruption in the signal transduction process linking the ligand-binding sites to the channel activation gate (38, 39). Given these findings, we hypothesized that the R271Q mutation may also affect the efficacy with which ivermectin is able to activate the channel. Although 0.03 μM ivermectin induced little direct activation of the R271Q mutant GlyR, it potentiated the magnitude of currents activated by a 7 mM (EC_{50}) concentration of glycine (Fig. 5*A*). Since this is similar to the effect of ivermectin on the WT GlyR, the mutation has apparently not altered the receptor sensitivity to low ivermectin concentrations. On the other hand, although the WT GlyR is strongly activated by 3 μM ivermectin, this concentration induced only a weak direct activation of the R271Q mutant GlyR (Fig. 5*B*). In addition, we were surprised to find that prior exposure of GlyRs to 3 μM ivermectin converted glycine into an irreversible agonist (Fig. 5*B*), an effect that is considered further below. When applied at a concentration of 30 μM, the ivermectin-gated current was 4.8 ± 1.2 ($n = 4$) times the magnitude of the current activated by a saturating (70 mM) glycine concentration (*e.g.* Fig. 5*C*). Once currents were maximally activated by ivermectin, a subsequent application of 7 mM glycine induced an additional small irreversible current (Fig. 5*C*). The mean glycine and ivermectin EC_{50} and n_H values for the R271Q mutant GlyR are given in Table I. The results indicate that although this mutation decreased the glycine sensitivity by a factor of 273, the ivermectin sensitivity was decreased only by a factor of 20. Taken together, these observations suggest that R271Q disrupted the glycine gating mechanism to a much greater extent than that of ivermectin. By comparison, it is relevant to note that the binding site mutations, Y202F and T202A, had no significant effect on the relative magnitude of ivermectin- *versus* glycine-gated currents (Table I), although their glycine sensitivity was similar to that of R271Q.

As indicated above, prior application of 3 μM ivermectin induced glycine to irreversibly increase the current magnitude. It is noteworthy that this irreversible "glycine-enhanced" current was much larger in magnitude than that induced by a saturating glycine concentration prior to ivermectin exposure

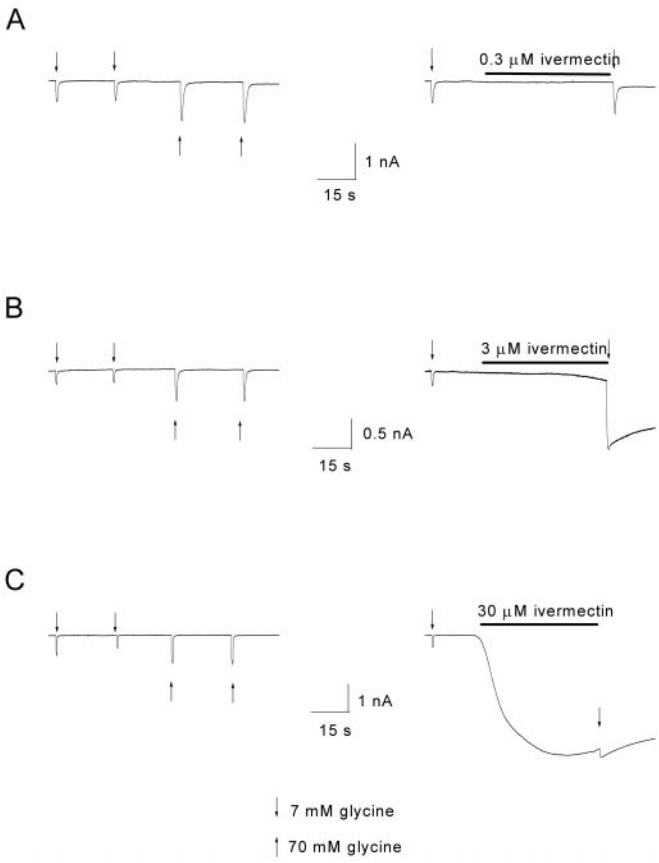

FIG. 5. **Effects of ivermectin on the R271Q mutant GlyR.** *A–C,* each cell was treated first with two applications of a half-saturating (7 mM) glycine concentration, followed by two applications of a saturating (70 mM) glycine concentration (*left panels*). Following this, ivermectin was applied at the indicated concentration, and glycine responses were recorded again (*right panels*). Note the potent irreversible activation by 7 mM glycine in *B* (*right panel*).

(Fig. 5*B*). In fact, the magnitude of current activated by 7 mM (EC_{50}) glycine was increased by 5.8 ± 2.1 ($n = 4$) times after exposure to 3 μM ivermectin. The pharmacology of this glycine-enhanced current was investigated by testing the effect of picrotoxin. In the WT GlyR, picrotoxin acts as a competitive antagonist of glycine-gated currents (28). However, in the R271Q mutant GlyR, picrotoxin is converted into a noncompetitive inhibitor of glycine-gated currents with an IC_{50} of 5 μM (28). When applied at a concentration of 1 mM, picrotoxin inhibited the irreversible glycine-enhanced current by 15 ± 7% ($n = 4$), indicating that this current is dramatically less picrotoxin-sensitive than the glycine-gated current. Given the weak picrotoxin sensitivity and the irreversible activation, this current more closely resembles the ivermectin-gated current than the glycine-gated current. The glycine exposure reduced the mean ivermectin EC_{50} value from 7.9 to 6.6 μM. These observations support the conclusion that a short application of glycine significantly increases the efficacy with which the prebound ivermectin can irreversibly activate the channels.

Effect of a Fast Desensitization Mutant on Ivermectin-gated

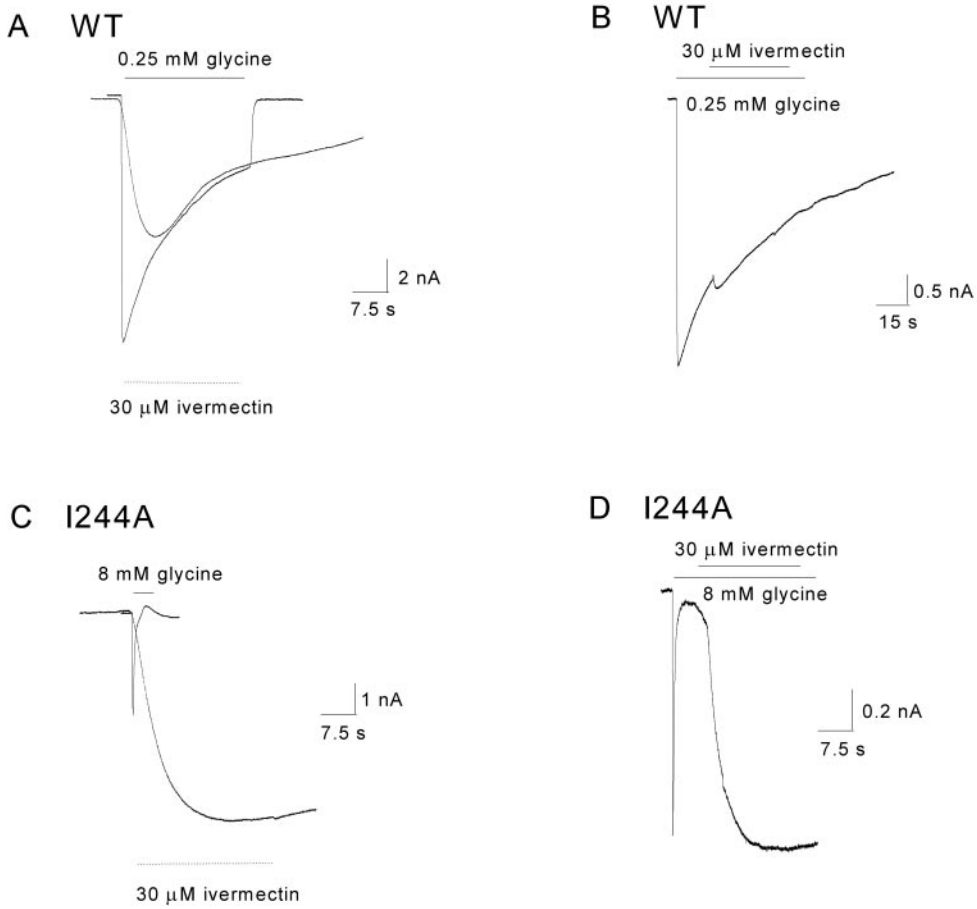

FIG. 6. **Comparison of the effects of ivermectin on the WT and I244A mutant GlyRs.** Traces in both *A* and *B* were recorded from WT GlyRs, whereas traces in *C* and *D* were recorded from I244A GlyRs. *A,* examples of WT GlyR currents activated by the indicated (saturating) concentrations of glycine and ivermectin in the same cell. *B,* ivermectin (30 μM) has little additional effect when applied to WT GlyRs already maximally activated by 250 μM glycine. *C,* examples of I244A mutant GlyR currents activated by the indicated (saturating) concentrations of glycine and ivermectin. Both traces were recorded from the same cell. Note the larger amplitude and the slower decay rate of the ivermectin-gated current. *D,* a saturating concentration of ivermectin is still able to irreversibly activate a large current even after complete desensitization of the I244A mutant GlyR to a saturating glycine concentration.

Responses—A final series of experiments was directed at determining whether a prolonged glycine exposure could induce cross-desensitization of ivermectin-gated currents. This experiment is difficult to perform using recombinantly expressed WT GlyR α_1 subunits because these display a slow rate of desensitization. Indeed, most of the observed decay of glycine-gated currents in whole cell recordings is due to chloride shift effects (38). However, since glycine-gated currents in the I244A mutant GlyR desensitize rapidly with a time constant of about 1 s (39), this mutant receptor provides a suitable model for investigating the effects of glycine-induced desensitization on ivermectin-gated currents.

In the WT GlyR, a saturating concentration (30 μM) of ivermectin induced a current that decayed at approximately the same rate as the glycine-gated current recorded in the same cell (Fig. 6*A*). Furthermore, when ivermectin was coapplied with glycine, the rate of current decay was not changed (Fig. 6*B*). Similar results were observed in four cells. As discussed below, these results contrast dramatically with those recorded from the I244A mutant GlyR.

Apart from its effects on desensitization, I244A also reduced both the glycine sensitivity and the agonist efficacy of β-alanine and taurine relative to glycine (39). The glycine and ivermectin EC_{50} and n_H values for the I244A mutant GlyR are displayed in Table I. The I244A mutation reduces the glycine sensitivity by a factor of 30, although the ivermectin sensitivity is not affected. The recovery time constant from the glycine-induced fully desensitized state to the resting closed state was measured to be 3.2 ± 0.49 s (n = 3). An example of a glycine-gated current recorded from a cell expressing the I244A mutant GlyR is displayed in Fig. 6*C*. The rapid rate of desensitization of this current is apparent. An example of an ivermectin-gated current recorded from the same cell 30 s later is also shown (Fig. 6*C*). It is apparent that ivermectin not only irreversibly activates this mutant GlyR but that the magnitude of the current activated by a saturating (30 μM) concentration of ivermectin is greater than that activated by a saturating (8 mM) concentration of glycine. Indeed, the ratio of the saturating ivermectin-*versus* glycine-gated current averaged from four cells was 1.7 ± 0.2 (n = 4), suggesting that the I244A mutation reduces glycine efficacy. This observation is consistent with a previous study that concluded that the mutation disrupted the glycine signal transduction mechanism (39).

As displayed in Fig. 6*D*, ivermectin potently activated the I244A mutant GlyR after the receptor was completely desensitized to glycine. The mean ratio of the saturating ivermectin-*versus* glycine-gated current after glycine-induced desensitization was 1.3 ± 0.2 (n = 4), which using a one-way ANOVA was not significantly different from that obtained before glycine-induced desensitization ($p > 0.05$). The rate of ivermectin-induced channel activation was not affected by the presence of glycine. The mean activation time constant of currents gated by 30 μM ivermectin in the presence of glycine was 5.3 ± 2.0 s (n = 4), whereas in the absence of glycine was 3.6 ± 0.6 s (n = 3). By

TABLE III
Comparison of glutamate- and ivermectin-PO_4-gated currents in various GluClRs

Receptor	Glutamate		Ivermectin-PO_4		Ivermectin potentiation of glutamate-gated currents	Ref.
	Agonist activity	Picrotoxin sensitivity	Agonist activity	Picrotoxin sensitivity		
C. elegans GluClα homomer	None	NA[a]	Irreversible EC_{50} = 0.14 μM	Low IC_{50} = 59 μM	NA	4, 41
C. elegans GluClβ homomer	Reversible EC_{50} = 380 μM	High IC_{50} = 0.1 μM	None	NA	None	4, 41
C. elegans GluCl$\alpha\beta$ heteromer	Reversible EC_{50} = 1360 μM	Low IC_{50} = 42 μM	Irreversible EC_{50} = 0.19 μM	Low IC_{50} = 52 μM	Strong (500%)	4, 41
C. elegans GluClα_2 homomer$_2$	Weak, reversible EC_{50} = 208μM	Very low IC_{50} >100 μM	Irreversible EC_{50} = 0.11 μM	Very low IC_{50} >100 μM	?	6
C. elegans GluCl$\alpha_2\beta$ heteromer	Reversible EC_{50} = 62 μM	Very low IC_{50} >100 μM	Irreversible EC_{50} = 0.10 μM	Very low IC_{50} >100 μM	?	6
Drosophila GluCl α homomer	Reversible EC_{50} = 23 μM	Very low IC_{50} > 500 μM	Irreversible EC_{50} = 0.04 μM	Very low IC_{50} > 500 μM	Weak (24%)	5

[a] NA, not applicable.

using a one-way ANOVA, these values are not significantly different ($p > 0.05$). These results demonstrate that ivermectin activates the I244A mutant GlyR after it has been completely desensitized to a prior glycine application. Furthermore, the ivermectin activation rate is not changed in the glycine-induced desensitized state.

DISCUSSION

Novel Mechanism of Ivermectin Activation—Ivermectin exerted dual effects on the WT GlyR. At low (0.03 μM) concentrations it potentiated the response to sub-saturating glycine concentrations, and at higher (≥0.03 μM) concentrations it directly activated GlyR Cl^- currents. These effects are similar to those of ivermectin on the *Caenorhabditis elegans* $\alpha\beta$ heteromeric GluClR and the *Drosophila* homomeric α GluClR (Table III). This functional similarity may reflect the close phylogenetic relationship between the GluClR and GlyR gene families (14). However, the results of the present study contrast with those recently found in GlyRs from cultured cortical neurons, where 1 μM ivermectin inhibited glycine-activated currents in a use-dependent manner, while simultaneously slowing the channel closing rate (10). Differences in ivermectin effects may depend on subunit composition (Table III), and since the subunit composition of the GlyR studied in (10) is undefined, it is not possible at present to resolve these differences.

Ivermectin also exerts a range of effects on some other members of the ligand-gated ion channel superfamily. For example, it reversibly activates recombinantly expressed $\alpha_1\beta_2$, $\alpha_1\beta_1\gamma_2$, $\alpha_1\beta_2\gamma_2$, $\alpha_1\beta_2\gamma_{2s}$, $\alpha_1\beta_3\gamma_2$, and $\alpha_1\gamma_{2s}$ $GABA_A$Rs (10, 18) as well as a GABA-mediated Cl^- current in dorsal root ganglion neurons (17). Low ivermectin concentrations (<0.1 μM) potentiate GABA-mediated Cl^- currents in hippocampal neurons (15), $GABA_A$Rs expressed from chick brain mRNA (16), as well as recombinantly expressed $\alpha_1\beta_1\gamma_2$, $\alpha_1\beta_2\gamma_2$, and $\alpha_1\beta_3\gamma_2$ $GABA_A$Rs (10). Finally, in recombinantly expressed α_7 homomeric nAchRs, 30 μM ivermectin enhances the acetylcholine-evoked current but displays no agonist activity (19). The recently reported potentiating effect of ivermectin on $P2X_4$ receptor channels (40) indicates that the effects of ivermectin are not limited to members of the ligand-gated ion channel superfamily.

The present study demonstrates that ivermectin-gated currents are relatively insensitive to picrotoxin (Table II). This contrasts with the effects of picrotoxin on the GluClR, where the picrotoxin sensitivity was similar for ivermectin- and glutamate-gated currents (Table III). The present study also found that the ivermectin-gated currents exhibited a dramatically reduced sensitivity to strychnine and zinc (Table II). The reduction in strychnine potency could not be explained by ivermectin preventing the access of strychnine to its binding site. The novel pharmacology of the ivermectin-induced activated state supports the conclusion that ivermectin activates the GlyR by a novel mechanism. In the α_7 nAChR, ivermectin potentiation also resulted in a modification of the receptor pharmacological profile (19).

In addition to activating the channels by a novel mechanism, it is likely that ivermectin binds to a novel, as yet unidentified, binding site. This study has shown that the mutation of known glycine-binding sites had very little effect on ivermectin sensitivity (Table I). In particular, the Y202F and T204A mutations, which strongly disrupted the apparent glycine affinity, had little effect on ivermectin sensitivity. Thus, although the ivermectin- and glycine-binding sites may overlap to some degree, the two molecules clearly do not bind to an identical set of contact sites. Since Tyr^{202} is both a glycine- and strychnine-binding site (26, 27), the observation that ivermectin does not bind to Tyr^{202} may explain the reduced strychnine sensitivity of the ivermectin-gated response.

Further evidence for a novel mechanism of action by ivermectin was sought by investigating whether it could activate a GlyR that had already been completely desensitized to glycine. As displayed in Fig. 6*D*, ivermectin strongly and irreversibly activated the I244A mutant GlyR after it had previously been completely desensitized to glycine, suggesting that it could return desensitized channels directly to the activated state. This strongly supports the previous conclusion that ivermectin activates the GlyR via a novel mechanism.

Allosteric Disruption of the Ivermectin Activation Mechanism—The mechanism of action of ivermectin was investigated further by examining the effect of the R271Q mutation. This mutation, which is a cause of human startle disease, has been shown to reduce the apparent affinities of the agonists glycine, β-alanine, and taurine (37, 38). It also converts taurine and β-alanine into competitive antagonists of glycine, without dramatically affecting their binding affinities (38). These effects are consistent with a model whereby R271Q completely disrupts the signal transduction pathway linking the taurine and β-alanine agonist-binding sites to the activation gate (39). Although the mutation appears to have a proportionately greater disruptive effect on the agonist efficacy of the relatively lower

affinity agonists, β-alanine and taurine, it also reduces the agonist efficacy of glycine (38, 39). This observation also suggests that since ivermectin activates the GlyR with a higher affinity than does glycine, the R271Q mutation should have a proportionately weaker disruptive effect on the ivermectin agonist transduction mechanism. Indeed, by demonstrating that the ivermectin sensitivity is little affected by R271Q and that the maximum ivermectin-gated current is about 5 times that activated by a saturating glycine concentration, this study demonstrates that the glycine agonist transduction mechanism is disrupted to a greater extent than that of ivermectin.

The I244A mutation causes relatively small reductions in the apparent agonist affinities of glycine, β-alanine, and taurine and results in β-alanine and taurine being converted into partial agonists relative to glycine (39). Thus, this mutation induces a similar, but relatively weaker, phenotype than that induced by R271Q. Hence, it is not surprising that the I244A mutation has little effect on ivermectin sensitivity (Table I). Furthermore, since the mutation disrupted the glycine signal transduction pathway to a relatively small extent (39), it may be expected that the ratio of the peak ivermectin-gated current to peak glycine-gated current would not be as large as it is for R271Q. Indeed, the mean ratio for the I244A mutant GlyR was 1.7, whereas the corresponding ratio for the R271Q mutant GlyR was 4.8 (Table I). Hence, the results are consistent with the idea that I244A also disrupts the ivermectin agonist transduction mechanism to a lesser extent than the glycine transduction mechanism.

Conclusion—This study concludes that ivermectin-gated currents in the GlyR are similar to those in some GluClRs in that they potentiate agonist responses at low concentrations and irreversibly activate the receptor at higher concentrations. However, the GlyR differs in that the ivermectin-gated currents have a different pharmacology to glycine-gated currents. Consistent with this observation, this study demonstrates that the ivermectin- and glycine-binding sites are not identical. In addition, the observation that ivermectin potently activates a mutant GlyR after its response to glycine is completely desensitized provides strong evidence for a novel mechanism of activation. Furthermore, mutations that are known to disrupt the agonist signal transduction mechanism have a relatively small effect on the ivermectin agonist transduction mechanism relative to those of glycine, β-alanine, or taurine. Together, these findings indicate that ivermectin activates the GlyR by a novel mechanism.

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