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The Glycine Receptor: Pharmacological Studies and Mathematical Modeling of the Allosteric Interaction Between the Glycine- and Strychnine-Binding Sites

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SUMMARY

The displacement by glycine of ^{3}H-strychnine binding to rat spinal cord membranes cannot be explained by a simple competitive interaction. Indeed, protein-modifying reagents can completely abolish the inhibition of ^{3}H-strychnine binding by glycine and other agonists, whereas the interaction of strychnine itself and other related compounds with the binding site is unimpaired. Moreover, glycine cannot inhibit completely saturable ^{3}H-strychnine binding, the extent of its maximum inhibitory effect depending on the ionic composition of the medium. Hill coefficients less than 1 (whose magnitude also depends on the assay medium) were obtained from glycine displacement curves. These properties are consistent with a mathematical model of two different, but mutually interacting, binding sites for strychnine and glycine on the glycine receptor. The effect of ions and protein-modifying reagents might be explained in this model as modifications of the mechanisms that mediate the allosteric interaction, and/or the affinity of glycine for the receptor. The agonists β-alanine and taurine and the new antagonists, THAZ, iso-THAZ, and 4,5-TAZA, also seem to interact with a site different from the strychnine-binding site, probably the glycine-binding site.

Glycine is a major inhibitory neurotransmitter in the spinal cord and other regions of the vertebrate nervous system (1–3). The binding of this amino acid to its receptor inhibits neuronal firing, and this effect is antagonized by the alkaloid strychnine. Radiolabeled strychnine has been used widely to study and localize the glycine receptor in the nervous systems of several different species (4–7). Strychnine has also been employed as a tool in the purification and biochemical characterization of the glycine receptor (8, 9).

^{3}H-Strychnine binding to spinal cord membranes is displaceable by a number of compounds including unlabeled strychnine and related compounds (10, 11), glycine and other receptor agonists (4–6), the γ-aminobutyric acid-A receptor antagonist bicuculline (12), the antihelmintic avermectin B_{1a} (13), and several benzodiazepines (14).

The lack of other suitable radioligands for the characterization of this receptor has always been a serious handicap. ^{3}H-Glycine occasionally has been used in binding experiments (15–18), but its ability to label specifically the glycine receptor remains uncertain. The interaction of glycine with central nervous system membrane preparations seems to be complex, possibly involving a heterogeneous population of binding sites (17) and/or cooperative interactions (18). Due to low affinity and lack of pharmacological specificity, it is not clear at the moment whether these binding sites are, in fact, on the glycine receptor or not.

Some experiments have suggested that strychnine and glycine have two different binding sites on the glycine receptor (5). Indeed, the inhibition of ^{3}H-strychnine binding by glycine is prevented by treatment with diazonium tetrazole or acetic anhydride. In the present work, we have used this test to investigate whether the site of action of different inhibitors of ^{3}H-strychnine binding is related to the strychnine-binding site or to the glycine-binding site. We have also studied the properties of the displacement curves of ^{3}H-strychnine binding by glycine and some other compounds, in order to characterize the interaction between the glycine- and strychnine-binding sites. The displacement of ^{3}H-strychnine binding by glycine and β-alanine can be fitted quite well to a mathematical model based

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ABBREVIATIONS: dimethyl-POPOP, 1,4-bis-(4-methyl-5-phenyl-2-oxazolyl)-benzene; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PK-8165, 2-phenyl-4-(2-(4-piperidinyl)ethyl)quinoline; Ro-15-1788, ethyl-8-fluoro-5,6-dihydro-5-methyl-6-oxo-4H-imidazo-(1,5-a)-(1,4)-benzodiazepine-3-carboxylate; 4,5-TAZA, 2,3,6,7-tetrahydro-1H-azepine-4-carboxylic acid; THAZ, 5,6,7,8-tetrahydro-4H-isoxazolo-(4,5-d)-azepin-3-ol; iso-THAZ, 5,6,7,8-tetrahydro-4H-isoxazolo-(3,4-d)-azepin-3-ol.

on an allosteric interaction between the strychnine-binding site and a different site for these inhibitors.

Materials and Methods

Preparation of membranes from rat spinal cord. Male Wistar rats (60 days old) were decapitated, and their medullae oblongatae were carefully dissected from the brains. The spine was cut in the lumbar region and the spinal cord was ejected by forcing compressed air into the cavity. Both tissues were pooled together and synaptic membranes were prepared by the method of Young and Snyder (5). In brief, P2 fractions were obtained and resuspended in 5 volumes of distilled water. Then, they were homogenized with a Polytron (set 3, 30 sec) and centrifuged at 8,000 × *g* for 20 min. The supernatant and the upper buffy coat were collected and centrifuged again at 48,000 × *g* for 20 min. The resulting pellet was resuspended in a minimum volume of distilled water or buffer (50 mM sodium-potassium phosphate, pH 7.1) and stored at −60° until used. Under these conditions, ^{3}H-strychnine binding was stable for at least 2 weeks.

Binding assay. The membranes were always stored for between 24 hr and 2 weeks. The membranes were thawed and resuspended to give a final protein concentration in the binding assay of 0.2–0.4 mg/1.2 ml. The binding assay was performed in a final volume of 1.2 ml of 50 mM sodium-potassium phosphate buffer, pH 7.1 with—unless stated otherwise—2 nM ^{3}H-strychnine. The tubes were incubated at 0–4° for 10 min and then 1 ml of the suspension was filtered through Whatman GF/B glass fiber filters, 25 mm diameter. The filters were rinsed immediately with 5 ml of 0.15 M NaCl and placed in 20-ml glass counting vials where they were dried at 100° for approximately 2 hr. The whole filtering and washing procedure took less than 10 sec.

Five ml of toluene:Triton X-100:2,5-diphenyloxazole:dimethyl-POPOP scintilliation cocktail were added to the vials with the filters, and the radioactivity was measured in a refrigerated liquid scintillation counter (Intertechnique-3B) with a counting efficiency for ^{3}H of 30%.

Specific ^{3}H-strychnine binding was defined as the binding displaceable by 0.1 mM unlabeled strychnine.

Treatment of membranes with protein-modifying reagents. *Diazonium tetrazole.* Spinal cord membranes (1 mg of protein/ml) were incubated at 4°, 60 min, in the presence of several concentrations of this reagent up to 2.5 mM. *Acetic anhydride.* Membranes (1 mg of protein/ml) were incubated for 30 min at 4° with different concentrations of acetic anhydride up to 50 mM. After both treatments, the membranes were washed twice with 50 mM sodium-potassium phosphate buffer, pH 7.1, at 4°, and collected by centrifugation (48,000 × *g*, 15 min). Membranes treated in this way were used immediately in the binding assay.

Protein determination. Membrane proteins were measured according to the method of Resch *et al.* (19).

Materials. ^{3}H-Strychnine (15–20 Ci/mmol) was purchased from Amersham. Avermectin B_{1a} was a gift from Dr. Leslie Iversen, Merck Sharp and Dohme Laboratories, Hoddesdon, Hertfordshire, England. Strychnine-*N*-oxide, *N*-methyl-strychnine, and α- and β-colubrine were gifts from Prof. K. Bernaver and Dr. H. Gutmann, Hoffman-La Roche, Basel, Switzerland. THAZ, iso-THAZ and 4,5-TAZA were gifts from Dr. P. Krogsgaard-Larsen, The Royal Danish School of Pharmacy, Copenhagen, Denmark. PK-8165 was a gift from Pharmuka Laboratories, Paris, France. Diazepam, flunitrazepam, and Ro-15-1788 were gifts from Hoffman-La Roche, Nutley, NJ. 2-Nitrostrychnine and 2-amino-strychnine were synthesized from strychnine sulfate in the Organic Chemistry Department, Universidad Autónoma de Madrid, by the methods of Rosenmund (20) and Tedeschi *et al.* (21), respectively. Diazonium tetrazole was prepared from 5-aminotetrazole (22, 23) purchased from Aldrich. All other chemicals were purchased from standard commercial sources.

Results

Compounds that inhibit ^{3}H-strychnine binding. The most potent compound found to displace ^{3}H-strychnine binding was strychnine itself. Other strychnine-related compounds were also potent displacers (Table 1), the potency of α-colubrine and 2-aminostrychnine being very close to that of strychnine.

^{3}H-Strychnine binding was also displaceable by the glycine receptor agonists glycine, β-alanine, taurine, and β-aminoisobutyric acid (Table 1). The potencies of glycine and β-alanine as inhibitors of ^{3}H-strychnine binding were very similar. The Hill coefficients for the displacement were close to unity for β-alanine and taurine under these conditions, and slightly, but significantly, less than unity for glycine (Table 1). It should be noted that with incubation media different from the 0.2 M NaCl, 50 mM sodium-potassium phosphate buffer habitually used, the Hill coefficient for β-alanine also tends to be <1, and an even smaller Hill coefficient for glycine is obtained (see Fig. 8). Glycine, β-alanine, and taurine inhibited ^{3}H-strychnine binding, decreasing its affinity without changing the B_{max} (Fig. 1).

A new family of glycine antagonists has been discovered recently by Krogsgaard-Larsen *et al.* (24). The potency of three of these compounds, namely, THAZ, iso-THAZ, and 4,5-TAZA, as displacers of ^{3}H-strychnine binding (Table 1) correlated well with their potency in inhibiting the hyperpolarizing action of glycine on cat spinal interneurons (24). It should be noted that iso-THAZ, together with avermectin B_{1a}, is the most potent displacer of ^{3}H-strychnine binding without strychnine-related structure.

Other ^{3}H-strychnine-binding displacers (Table 1) include several benzodiazepines, the benzodiazepine receptor partial agonist PK-8165 (25), the γ-aminobutyric acid-A receptor antagonist bicuculline, and the anthelmintic avermectin B_{1a}. PK-8165 was 1 order of magnitude more potent (IC_{50} 8.2 μM) than

TABLE 1

Inhibition of ^{3}H-strychnine binding by different compounds

Binding assays were conducted with 2 nM ^{3}H-strychnine in the presence of 0.2 M NaCl, 50 mM sodium-potassium phosphate buffer, pH 7.1, at 4°. IC_{50} and n_H (Hill coefficient) values were calculated by least squares fitting of Hill plots obtained from displacement curves with at least five different concentrations of inhibitor. Values are the mean ± standard error of at least three determinations. The following compounds did not inhibit ^{3}H-strychnine binding or had IC_{50} values higher than 1 mM: D-serine, proline, *N*-toluyl-glycine, dimethylglycine, L-kinurenine, isoquinolinic acid, picrotoxin, and pentobarbital.

Inhibitor	IC_{50}	n_H
Strychnine	7.9 ± 2.2 nM	0.97 ± 0.03
2-Aminostrychnine	30 ± 11 nM	0.93 ± 0.18
α-Colubrine	23 ± 7 nM	0.97 ± 0.09
β-Colubrine	177 ± 51 nM	0.92 ± 0.07
Brucine	101 ± 47 nM	0.67 ± 0.01
N-Methyl-strychnine	652 ± 73 nM	0.97 ± 0.12
2-Nitro-strychnine	660 ± 54 nM	1.03 ± 0.08
Strychnine-*N*-oxide	3.5 ± 0.3 μM	
Glycine	32 ± 6 μM	0.76 ± 0.04
β-Alanine	38 ± 9 μM	1.02 ± 0.08
Taurine	67 ± 14 μM	1.00 ± 0.05
β-Aminoisobutyric acid	268 ± 43 μM	0.89 ± 0.19
THAZ	62 ± 5 μM	0.81 ± 0.14
iso-THAZ	2.8 ± 0.8 μM	0.67 ± 0.04
4,5-TAZA	3.7 ± 1.2 μM	0.87 ± 0.05
PK-8165	8.2 ± 1.2 μM	1.02 ± 0.04
(+)-Bicuculline	6.2 ± 0.8 μM	0.82 ± 0.10
Avermectin B_{1a}	1.0 ± 0.3 μM	0.50 ± 0.11

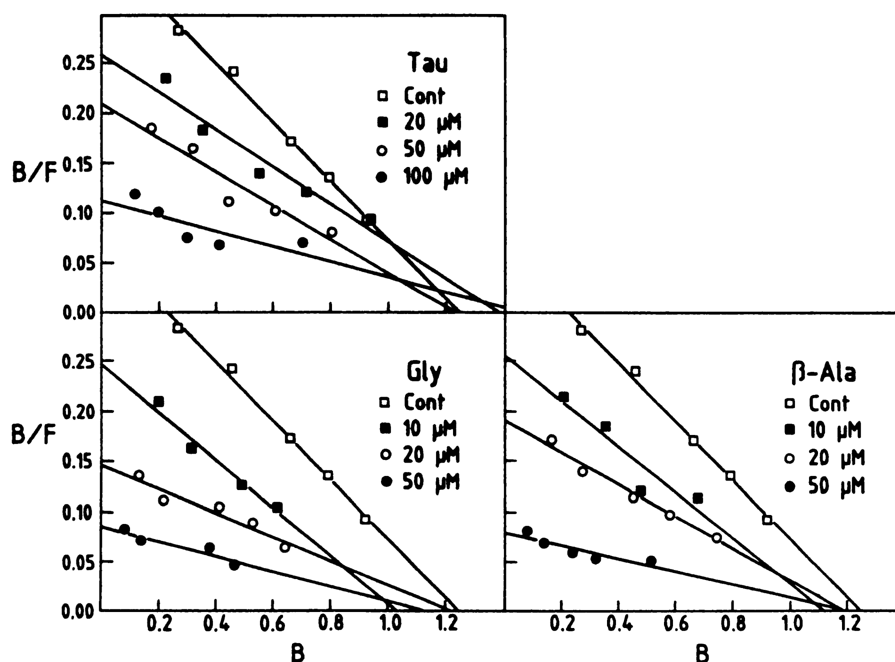


Fig. 1. Scatchard plots of ^{3}H-strychnine binding in the presence of glycine, β-alanine, and taurine. Spinal cord membranes were incubated with several concentrations of ^{3}H-strychnine in 0.2 M NaCl, 50 mM sodium-potassium phosphate buffer, pH 7.1, at 4°, alone or in the presence of glycine (*Gly*), β-alanine (β-Ala), or taurine (*Tau*) at the concentrations indicated. Lines drawn represent a least squares fit of the experimental data. *B* is pmol of ^{3}H-strychnine specifically bound/mg of protein, *F* is concentration of free ^{3}H-strychnine in nM. Each determination was made in triplicate.

the benzodiazepines to inhibit ^{3}H-strychnine binding. The displacement by this compound had a Hill coefficient close to unity and was complete at 0.1 mM PK-8165 (Fig. 2). By contrast, the Hill coefficients for (+)-bicuculline and avermectin B_{1a} were 0.8 and 0.5, respectively, reflecting a complex interaction of these compounds with the glycine receptor. Moreover, despite its low IC_{50} value, avermectin B_{1a} was not able to inhibit completely ^{3}H-strychnine binding, its displacement curve reaching a plateau at high concentrations (Fig. 2).

Effect of inhibitors on K_D and B_{max}. Nearly all of the compounds tested inhibited ^{3}H-strychnine binding, lowering the affinity for the ligand without affecting its B_{max} (Figs. 1 and 3), this being called usually "competitive" inhibition. The only compounds that seem to lower the B_{max} of ^{3}H-strychnine binding were avermectin B_{1a} (Fig. 3), (+)-bicuculline (Fig. 3), and NH_4Cl (26). Avermectin B_{1a} decreased the B_{max} without apparently changing the K_D ("noncompetitive" inhibition), whereas a small change in K_D was observed with the higher concentration of bicuculline used.

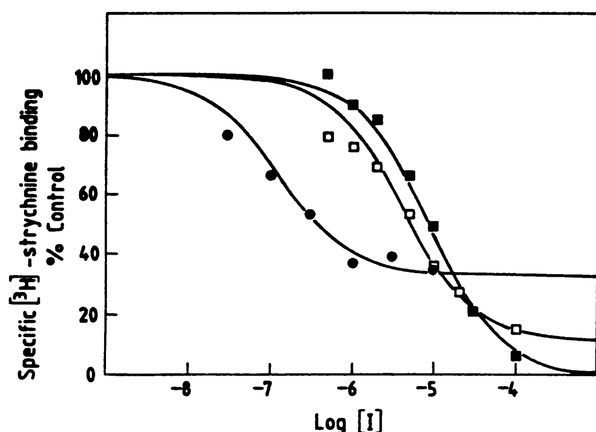


Fig. 2. Displacement of ^{3}H-strychnine binding by PK-8165, (+)-bicuculline, and avermectin B_{1a}. Spinal cord membranes were incubated with 2 nM ^{3}H-strychnine in 0.2 M NaCl, 50 mM sodium-potassium buffer, pH 7.1, at 4°, in the presence of increasing concentrations of PK-8165 (■), (+)-bicuculline (□), or avermectin B_{1a} (●). Sigmoidal lines fitting the points were created by an iterative computer program. A different method (Hill plot) was used to calculate the IC_{50} and n_H values given in Table 1. Points are the mean of three determinations. The experiment was replicated twice.

Experiments with protein-modifying reagents. Young and Snyder (5) demonstrated that, when the synaptic membranes were treated with somewhat low concentrations of the protein-modifying reagents diazonium tetrazole and acetic anhydride, the ability of 1 mM glycine, but not 0.1 mM strychnine, to inhibit ^{3}H-strychnine binding was eliminated. This result suggested that glycine and strychnine may bind to different parts of the glycine receptor, and that either the glycine-binding site or the allosteric interaction between the glycine- and the strychnine-binding sites were destroyed by these reagents. In order to know how other compounds interact with the glycine receptor, we have studied whether the same treatment can abolish the inhibition of ^{3}H-strychnine binding by these compounds (Fig. 4). The agonists glycine, β-alanine, and taurine could no longer inhibit ^{3}H-strychnine binding in membranes treated with either diazonium tetrazole or acetic anhydride, whereas the displacement by strychnine or the strychnine-related compounds brucine and 2-aminostrychnine was much less affected.

The most striking finding in these experiments was that the antagonists THAZ, iso-THAZ and 4,5-TAZA seem to interact with the glycine receptor more like glycine than like strychnine (Fig. 4): the fact that the inhibitory effect of these compounds on ^{3}H-strychnine binding was also abolished by diazonium tetrazole or acetic anhydride treatment indicates at least that they do not interact with the strychnine-binding site itself.

Conversely, PK-8165 does seem to bind to, or close to, the strychnine-binding site. The GABA antagonist (+)-bicuculline behaves like strychnine with respect to the effect of diazonium tetrazole, although this compound cannot bind to the strychnine-binding site itself because it is not a "competitive" inhibitor in saturation experiments (Fig. 3).

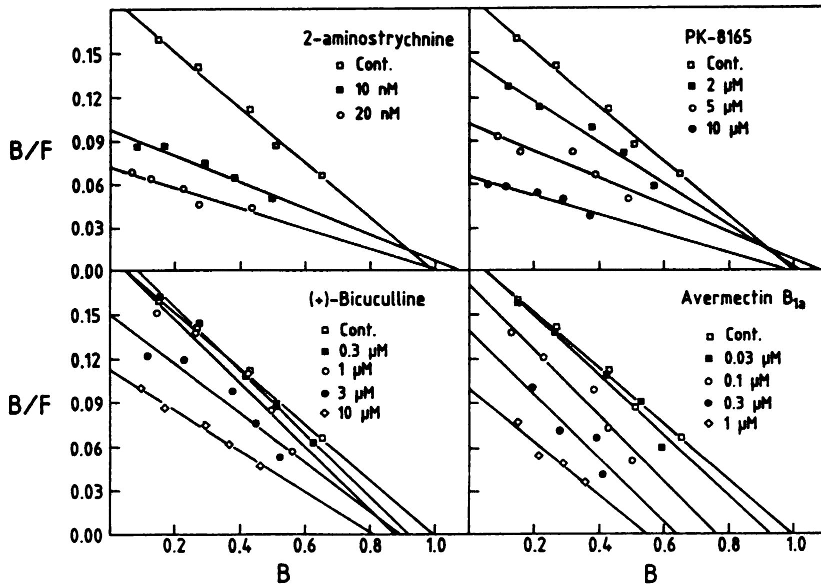


Fig. 3. Scatchard plots of ^{3}H-strychnine binding in the presence of 2-aminostrychnine, PK-8165, (+)-bicuculline, and avermectin B_{1a}. Experimental conditions and fitting of the points are as in Fig. 1.

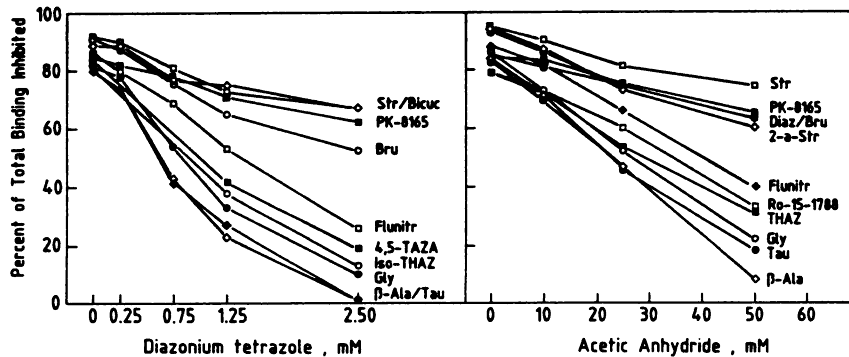


Fig. 4. Inhibition by several compounds of total ^{3}H-strychnine binding to membranes treated with diazonium tetrazole or acetic anhydride. Spinal cord membranes were treated with various concentrations of diazonium tetrazole (*left*) or acetic anhydride (*right*) as indicated in Materials and Methods. Treated membranes were then incubated with 2 nM ^{3}H-strychnine in 0.2 M NaCl, 50 mM sodium-potassium phosphate buffer, pH 7.1 at 4°, alone or in the presence of the following compounds: 0.1 mM cold strychnine (*Str*), 0.1 mM (+)-bicuculline (*Bicuc*), 0.1 mM PK-8165, 0.1 mM brucine (*Bru*), 0.1 mM 2-aminostrychnine (*2-a-Str*), 1 mM diazepam (*Diaz*), 1 mM flunitrazepam (*Flunitr*), 1 mM Ro-15-1788, 1 mM THAZ, 0.1 mM iso-THAZ, 0.1 mM 4,5-TAZA, 10 mM glycine (*Gly*), 10 mM β-alanine (*β-Ala*), or 10 mM taurine (*Tau*). Each point was determined in triplicate and represents the fraction of total binding inhibited by the compound.

The interaction of benzodiazepines with the glycine receptor remains unclear in these experiments. The inhibition of ^{3}H-strychnine binding by flunitrazepam and Ro-15-1788 is somewhat affected by acetic anhydride treatment, but the inhibition by diazepam is not. Perhaps this fact reflects a nonspecific interaction of benzodiazepines with the glycine receptor, or heterogeneity among the benzodiazepines in their mode of interaction with this receptor.

Effect of ions on the inhibition of ^{3}H-strychnine binding by glycine. The inhibition of ^{3}H-strychnine binding by glycine was affected not only by covalent modification of the glycine receptor by diazonium tetrazole or acetic anhydride, but also by changing the ionic composition of the medium that surrounds the receptor. As it has been previously reported (26–28), several ions including the Eccles' anions, akali metal cations, ammonium and choline, and also MOPS buffer, affect in different ways the properties of ^{3}H-strychnine binding.

The fraction of total ^{3}H-strychnine binding inhibited by 10 mM glycine was slightly smaller than the fraction inhibited by 0.1 mM strychnine when assayed in the usual NaCl/phosphate buffer (Fig. 5A). This difference was increased if 0.2 M NaCl was not included in the incubation medium (Fig. 5B). Even smaller fractions of total ^{3}H-strychnine binding were displaceable by glycine if ammonium phosphate (Fig. 5C), Tris-citrate (Fig. 5D), MOPS-Na^+ (Fig. 5E), or MOPS-NH_4^+ (Fig. 5F) buffers substituted for the sodium-potassium phosphate buffer. In all of these conditions the fraction of total binding displaceable by strychnine remained unaffected.

A possible explanation for these results would be the existence of strychnine-binding sites unrelated to the glycine receptor. Since total binding was almost the same in all of these conditions (28), except for the activation produced by NaCl in Fig. 5A, the former hypothesis would imply that "unrelated sites" were appearing and "glycine receptor sites" were disappearing when the buffer was changed. A more plausible explanation for these findings would be that the coupling between the binding sites for glycine and strychnine at the receptor depends on the composition of the medium.

The partial inhibition model. If both the ligand and the inhibitor interact with the same site at the receptor, then only two complexes can be formed, that is, receptor-ligand and receptor-inhibitor. This is called simple competitive inhibition, and will yield Hill plots with a slope of 1 from displacement curves.

In the case of allosteric inhibition, there will be two different binding sites for the ligand and for the inhibitor at the receptor molecule. The binding of each compound will inhibit the binding of the other compound by decreasing the affinity of the

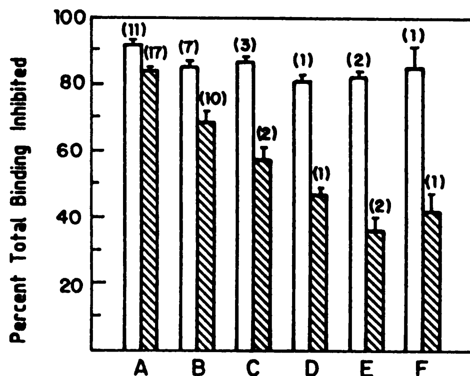
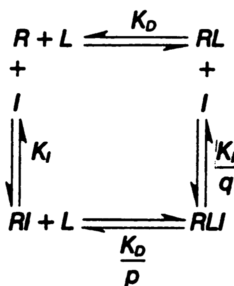


Fig. 5. Inhibition of total ^{3}H-strychnine binding by cold strychnine and glycine in different media. Each column represents the fraction of total ^{3}H-strychnine binding inhibited by 0.1 mM cold strychnine (□) or 10 mM glycine (▨). Incubation media are as follows: A, 0.2 M NaCl, 50 mM sodium-potassium phosphate; B, 50 mM sodium-potassium phosphate; C, 50 mM ammonium phosphate; D, 50 mM Tris-citrate; E, 50 mM MOPS-Na$^+$; F, 50 mM MOPS-NH$_4^+$. The pH was adjusted to 7.1 at 4° in each case. *Error bars* represent the mean ± standard error of the number of experiments indicated above in parentheses, except D and F, which represents a single experiment done in triplicate.

receptor for it. If this decrease in affinity is not strong enough to completely abolish the binding of the ligand, it is possible that the formation of a ternary complex, receptor-ligand-inhibitor, is the result of ligand binding to the receptor-inhibitor complex, or inhibitor binding to the receptor-ligand complex. Allosteric inhibition will then result from an increase in the equilibrium dissociation constant for the ligand (K_D) or for the inhibitor (K_i), by factors of $1/p$ and $1/q$, respectively ($0 < p \leq 1, 0 < q \leq 1$),



where R = receptor, L = ligand, I = inhibitor, RL = receptor-ligand complex, RI = receptor-inhibitor complex, RLI = receptor-ligand-inhibitor complex, K_D = equilibrium dissociation constant for the ligand, K_i = equilibrium dissociation constant for the inhibitor, and p and q = "partiality" parameters.

At equilibrium, the concentration of receptor-ligand-inhibitor complex will be the same no matter how it is formed, implying that $p = q$. We have termed this parameter p "partiality."

The amount of ligand bound to the receptor in the presence of inhibitor will be

$$B_I = \frac{B_{max} \cdot [L]}{K_D \cdot \dfrac{1 + \dfrac{[I]}{K_I}}{1 + p \cdot \dfrac{[I]}{K_I}} + [L]} \qquad (1)$$

Therefore, Scatchard plots in the presence of different concentrations of inhibitor will yield straight lines converging to the same value of B_{max} with different slopes.

The best way to find the different parameters—K_D, K_I, and p—of this model from experimental data is by changing the concentration of inhibitor, i.e., displacement experiments. Displacement curves corresponding to different values of p have been generated in Fig. 6 from Eq. 1, $10^{\mathrm{Log}\,[I]}$ substituting for $[I]$, where Log $[I]$ is now the independent variable.

Hill plots from the present model will yield curved lines (Fig. 7), corresponding to the following equation:

$$\mathrm{Log}\left(\frac{B_I}{B_O - B_I}\right) = \mathrm{Log}\left(\frac{K_I}{[I]} + p\right) + \mathrm{Log}\left(\left(1 + \frac{[L]}{K_D}\right) \cdot \left(\frac{1}{1 - p}\right)\right) \qquad (2)$$

where B_O and B_I represent binding in the absence of inhibitor and in the presence of a concentration $[I]$ of inhibitor, respectively.

The p (partiality) parameter of this model will describe

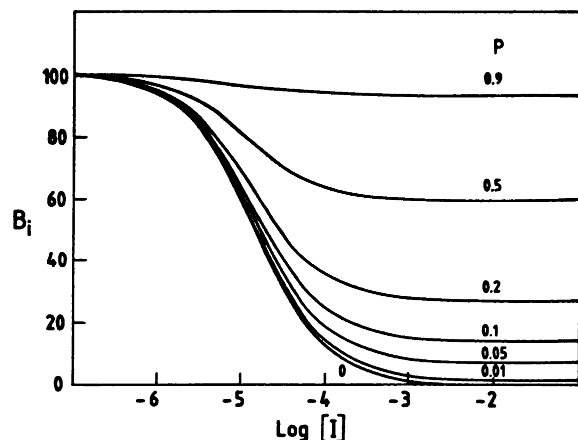


Fig. 6. Displacement curves generated by different p values in the partial inhibition model. The curves were generated from Eq. 1 by a computer program. Parameters of Eq. 1 were $(L) = 2.10^{-9}$M, $K_D = 4.10^{-9}$M, $K_I = 10^{-5}$M, $B_{max} = 300$, and those indicated for p. B_I and B_{max} are expressed as percentage of B_O, the amount of ligand bound in the absence of inhibitor.

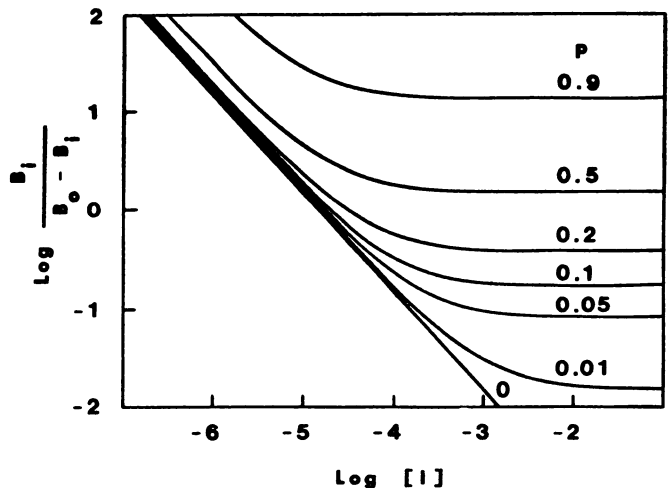


Fig. 7. Hill plots generated by different p values in the partial inhibition model. The curves were generated from Eq. 2 by a computer program. Parameters of Eq. 2 were $(L) = 2.10^{-9}$M, $K_D = 4.10^{-9}$M, $K_I = 10^{-5}$M, and those indicated for p.

specifically the strength of the allosteric inhibitory interaction between the ligand and the inhibitor-binding sites at the receptor molecule, whereas K_I will refer to the affinity of the inhibitor for its binding site. We assume $0 < p \leq 1$; as p approaches 1 the inhibitory effect becomes smaller (Fig. 6). In contrast, if $p > 0$, the affinity of the ligand for the RI complex becomes very small, and the system is indistinguishable from simple competitive inhibition. If $p = 1$, there is no inhibition, and if $p > 1$, there would be activation instead of inhibition, with an increased affinity of the ligand for its binding site at the receptor.

Thus, a compound would be a partial inhibitor if it fulfills the following criteria: (a) it can only inhibit a fraction of specific radioligand binding no matter how high its concentration is; (b) displacement curves with this inhibitor yield Hill coefficients less than unity (indeed, Hill plots are upward concave curves when carefully analyzed); and (c) Scatchard plots of saturation experiments in the presence of increasing concentrations of inhibitor reveal changes in the K_D with no changes in the B_{max} for the radioligand.

Application of the partial inhibition model to the interpretation of displacement experiments. The partial inhibition model shows how the incomplete inhibition of ^{3}H-strychnine binding by high (10 mM) concentrations of glycine can be explained as the result of an allosteric interaction between separate glycine- and strychnine-binding sites on the glycine receptor. This model also accounts for Hill coefficients less than 1 obtained from the displacement by glycine and β-alanine, and explains why these coefficients changed with the ionic composition of the medium. Indeed, Hill plots derived from this model are, in fact, upward concave curves, although points in the middle of these curves can be fitted to a straight line with a slope less than unity. Fig. 8 shows that when points away from the IC_{50} value are considered, curves are obtained that fit the equations of this model. Curved Hill plots were obtained for glycine (Fig. 8) and β-alanine (Fig. 9), which are expected to interact allosterically with ^{3}H-strychnine binding, based on the experiments with protein-modifying reagents (Fig. 4). In contrast, PK-8165, a compound with no strychnine-related structure, behaved like strychnine in this model (i.e., simple competition) (Fig. 8) and in the experiments with protein-modifying reagents (Fig. 4).

The strength of the allosteric interaction in the partial inhibition model is given by the coefficient, p. The bigger the p value is, the weaker is the allosteric interaction. Table 2 shows that changes in the ionic composition of the incubation media resulted mainly in changes in the parameter, p, but also in changes in K_D (equilibrium dissociation constant for strychnine) and K_I (equilibrium dissociation constant for the inhibitor). Thus, according to these facts, the glycine receptor can exist in different conformations depending on its ionic environment. Switching from one conformation to another implies changes in both the strychnine- and the glycine-binding sites, as well as in the allosteric coupling between them.

The interaction of avermectin B_{1a} with the glycine receptor is, however, more complex. Although the displacement curves for this compound against ^{3}H-strychnine binding do resemble what would be predicted by the partial inhibition model, the fact that avermectin B_{1a} appears to lower the B_{max} and not the K_I, for ^{3}H-strychnine cannot be reconciled with this model. Therefore, a third mechanism of interaction, unlike strychnine or glycine, would have to be proposed for this compound.

The displacement of ^{3}H-strychnine binding by glycine and β-alanine to membranes treated with acetic anhydride also seems to fit the partial inhibition model. Fig. 9 shows the Hill plots corresponding to these experiments. The main effect of the acetic anhydride treatment seems to be on the p coefficient, although this was not apparent at low concentrations (10 mM) of reagent. There were also some changes in K_D and K_I, which were decreased by 10 mM acetic anhydride and increased by 25 mM acetic anhydride (data not shown).

Discussion

The inability of ^{3}H-glycine to label specifically the glycine receptor makes the displacement of ^{3}H-strychnine binding by cold glycine the only method available to study the glycine-binding site on the receptor. The interaction of several other compounds with the glycine receptor can also be characterized in ^{3}H-strychnine-binding displacement experiments.

Glycine and strychnine do not seem to share the same binding site on the glycine receptor but have two different binding sites that interact allosterically, as was first demonstrated by experiments with protein-modifying reagents (5). In this paper we show that these reagents can also be used to differentiate compounds that bind to the strychnine-binding site from those that do not. Strychnine derivatives and PK-8165 seem to bind to the strychnine site, whereas the agonists glycine, β-alanine, and taurine bind to the glycine site. A striking result is that the compounds THAZ, iso-THAZ, and 4,5-TAZA also behave like glycine, possibly indicating that they bind to, or close to, the glycine-binding site, even though these compounds are antagonists (24).

In a previous study, Young and Snyder (5) found Hill coefficients greater than unity for displacement curves of ^{3}H-strychnine binding by glycine. These results were interpreted as a positive cooperative interaction between two or more molecules of glycine to displace one molecule of strychnine. However, we have not been able to find such Hill coefficients >1 in a wide range of experimental conditions. Instead, the slope of Hill plots from glycine displacement experiments in our hands were consistently <1, especially in experiments in which NaCl was not present in the medium. The reason for this discrepancy is not clear, but perhaps it could be due to modification of the receptor during the membrane preparation.

The partial inhibition model describes a system in equilibrium consisting of one population of receptors that bind two different compounds at two different binding sites. In this model, the binding of one compound at one site lowers the affinity of the receptor for the other compound at its site and the converse, but there is always the possibility that both compounds can bind to the receptor simultaneously. This model provides a good description of the displacement of ^{3}H-strychnine binding by glycine and other compounds as it is able to explain the following observations: (a) absence of complete inhibition of saturable ^{3}H-strychnine binding by glycine and other ligands, (b) slopes <1 in the Hill plots obtained from displacement curves; (c) effect of the ionic composition of the medium and protein reagents on the maximum inhibition and Hill plot slope of glycine and other compounds.

Hill plot slopes <1 are usually interpreted as negative cooperativity or receptor heterogeneity. However, it must be noted that the interpretation of Hill plots may not be the same when they are derived from displacement experiments instead of

Fig. 8. Displacement of ^{3}H-strychnine binding by glycine and PK-8165 in different media. Spinal cord membranes were incubated with 2 nM ^{3}H-strychnine in the presence of increasing concentrations of glycine (*Gly*) (*left*) or PK-8165 (*right*) in the following media: □, 0.2 M NaCl, 50 mM sodium-potassium phosphate; ■, 50 mM sodium-potassium phosphate; ○, 50 mM MOPS-Na. Each medium was adjusted to pH 7.1 at 4°. Experimental points were fitted to Eq. 1 of the partial inhibition model by an iterative computer program. The parameters thus obtained are summarized in Table 2. Displacement curves (*upper*) and curves in Hill plots (*lower*) were drawn with the calculated parameters by a computer using Eq. 1 or Eq. 2, respectively. Each *point* represents the mean ± standard error of three determinations; the experiment was repeated once.

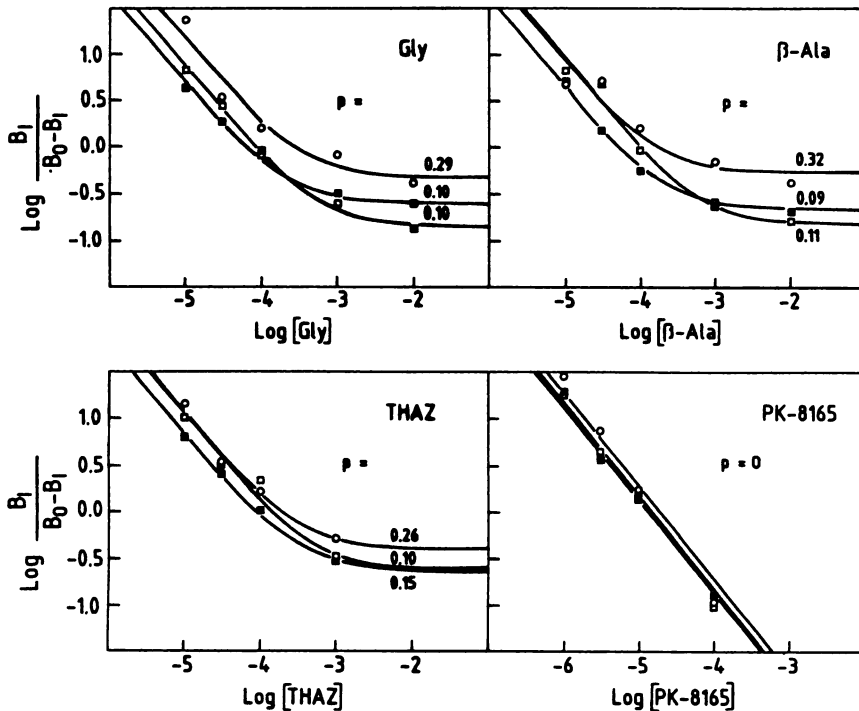


Fig. 9. Displacement by glycine, β-alanine THAZ, and PK-8165 of ^{3}H-strychnine binding to membranes treated with acetic anhydride. Spinal cord membranes were treated with acetic anhydride as indicated in Materials and Methods. Concentrations of acetic anhydride were: □, none; ■, 10 mM, ○, 25 mM. Treated membranes were then incubated with 2 nM ^{3}H-strychnine in 0.2 M NaCl, 50 mM sodium-potassium phosphate buffer, pH 7.1, at 4°, alone or in the presence of different concentrations of inhibitors. Experimental points were used to calculate the parameters of Eq. 1 of the partial inhibition model by an iterative computer program. Calculated parameters were then used to draw the curves from Eq. 2. Values of *p* corresponding to each curve are summarized in the figures. Each point represents the mean of three values.

saturation experiments. Indeed, a theoretical situation in which several molecules of inhibitor interact with one molecule of receptor to displace the ligand yields curved Hill plots convexed upward, regardless of whether there is positive, negative, or no cooperativity between the inhibitor-binding sites (29). These curved Hill plots would appear to have slopes >1. When there is strong negative cooperativity, these curves approach a straight line with a slope of 1, but never <1. Thus, negative cooperativity for the inhibitor cannot be the explanation for the Hill slopes <1 that we have found.

A theoretical system consistent with heterogeneous and not interconvertible binding sites with different affinities for the inhibitor also yields curved (sigmoidal) Hill plots. Like "partial inhibition," these curves may be taken for straight lines with a slope <1. In fact, a model based on heterogeneous receptors also assumes different binding sites for glycine and strychnine, which is the only basic assumption made in the partial inhibition model, and is supported by the experiments with protein-modifying reagents. Although heterogeneity of receptors remains an alternative explanation for our findings, we think that the effect of the medium on the displacement of ^{3}H-strychnine binding by glycine cannot be as easily explained with such a model.

Inhibition of ^{3}H-strychnine binding by avermectin B_{1a} and

TABLE 2

Parameters of the partial inhibition model calculated from displacement of ³H-strychnine binding by several compounds in different media

Displacement experiments were conducted as stated in Fig. 8. Incubation media were: 0.2 M NaCl, 50 mM sodium-potassium phosphate (NaCl/phosphate); 50 mM sodium-potassium phosphate (phosphate), and 50 mM sodium-MOPS (MOPS); in all cases pH was 7.1 at 4°. Parameters for the partial inhibition model were calculated from displacement curves having five to eight different concentrations of inhibitors, in triplicate, using Eq. 1. A Gauss iterative computer program was used in which p and K_I were allowed to vary, whereas K_D was constrained to the indicated values. These K_D values were obtained from saturation experiments done under the same conditions. B_{max} was calculated from control values (B_O) using Eq. 1. In some of the cases, iterations were possible allowing K_D also to vary, and the values of K_D, K_I, and p found this way were similar to those summarized here. Interestingly, for PK-8165 the p value was also constrained to 0 in a second iteration to improve the accuracy of the fitting. Minimization of the residual sum of squares of the deviations from the experimental points to the theoretical line was taken as an estimation of the goodness of fit.

Inhibitor	Medium	K_D, nM	K_I, μM	p
Gly	NaCl/phosphate	4	7,8	0,13
Gly	phosphate	14	10,9	0,50
Gly	MOPS	14	29,8	0,62
β-Ala	NaCl/phosphate	4	10,7	0,10
β-Ala	phosphate	14	18,7	0,31
β-Ala	MOPS	14	32,5	0,56
PK-8165	NaCl/phosphate	4	4,6	0
PK-8165	phosphate	14	13,9	0
PK-8165	MOPS	14	17,4	0

bicuculline cannot be explained by the partial inhibition model, since it requires inhibition to be due exclusively to a decreased affinity for the ligand, and significant changes in the the B_{max} for ³H-strychnine were found in the presence of both compounds.

Recently, Pfeiffer *et al.* (8) have purified the glycine receptor. It has three subunits (48, 58, and 93 kDa) and it is able to bind ³H-strychnine in a glycine-sensitive fashion. Photoaffinity labeling experiments (30, 31) have demonstrated that the strychnine-binding site is on the 48-kDa polypeptide. It is, however, unknown whether the glycine-binding site is on the same or another polypeptide. The second possibility is perhaps suggested by the high sensibility of the interaction between strychnine and glycine to changes in the ionic environment.

In this paper, we present further evidence for the existence of different but interacting binding sites for strychnine and glycine on the glycine receptor. The interaction between both sites can be described by a model that assumes the existence of a ternary complex between glycine, strychnine, and the receptor, whose stability depends on its ionic environment. A high affinity radioligand specific for the glycine-binding site would be necessary in order to completely characterize this ternary state.

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