0026-895X/88/060806-08$02.00/0

MOLECULAR PHARMACOLOGY, 34:806-813

Anion Regulation of [^{3}H]Strychnine Binding to Glycine-Gated Chloride Channels Is Explained by the Presence of Two Anion Binding Sites

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Received April 28, 1988; Accepted September 12, 1988

SUMMARY

The effects of six monovalent anions (chloride, bromide, iodide, nitrate, perchlorate, and thiocyanate) on [^{3}H]strychnine binding to glycine-gated chloride channels were examined. These anions have previously been shown to permeate glycine-gated chloride channels and stimulate [^{3}H]strychnine binding. Whereas low concentrations (10–200 mM) of all these anions enhanced [^{3}H] strychnine binding, higher concentrations (0.2–3 M) of thiocyanate, perchlorate, and iodide produced a robust inhibition of radioligand binding, and a more modest inhibition was observed with the same concentrations of nitrate and bromide. The presence of one binding site for anions at glycine-gated chloride channels can account for either the activation or the inhibition phase, but not both. However, these biphasic effects can be explained by the presence of two binding sites for anions at these channels. Two models with two anion binding sites were considered, the first assuming both allosteric activation and inhibition of the binding of the ligand, and the other explained by allosteric activation combined with competitive inhibition. Mathematical expressions for both models were formulated, and the equations obtained yielded satisfactory fitting to the results obtained with all anions tested in both concentration-response and saturation experiments. These equations also permitted the calculation of several parameters describing the interaction of the anions with these channels. The main difference in the behavior of these anions relates to the extent to which they produce activation of [^{3}H]strychnine binding and to their cooperative interaction at the two putative anion binding sites. Thus, a strong negative cooperativity was observed for the simultaneous binding of two molecules of chloride, bromide, or nitrate, but not for the simultaneous binding of thiocyanate, perchlorate, or iodide. This latter property may be related to the conductance of these anions through glycine-gated chloride channels.

Glycine is the primary mediator of synaptic inhibition in the spinal cord and brain stem (1, 2). Although glycine-induced hyperpolarization is due to a selective increase in chloride current under physiological conditions, the membrane permeability to several other small anions is also enhanced by this neurotransmitter (3–6). This increase in membrane permeability to anions has been explained by the existence of selective ion channels that open upon binding of glycine to its specific membrane receptor.

Subsequent electrophysiological experiments (7, 8) strongly suggest that glycine-gated chloride channels are not simply water-filled pores but entities containing specific binding sites that interact with anions by means of weak electrostatical forces (9, 10). This conclusion was supported by the following findings (7): (a) the permeability sequence of glycine-gated chloride channels for small monovalent anions differs from the sequence of their free-solution mobilities; (b) the conductance sequence of a series of anions is in the reverse order of their permeability sequence; (c) the conductance of these channels is saturable at high chloride and thiocyanate activities. Bormann *et al.* (7) have recently suggested that glycine-gated chloride channels possess two binding sites for anions. This conclusion was based on the observation that fitting current-voltage (I-V) curves for thiocyanate to a single-site model yielded affinity values for this anion that differed by 1 order of magnitude from its affinity deduced from conductance-activity relations. Moreover, the dependence of single-channel conductance on the proportion of thiocyanate in symmetrical chloride/thiocyanate mixtures was biphasic and could be fitted by a two-site, but not a single-site, model.

Biochemical studies also suggest the presence of anion binding sites at glycine-gated chloride channels. Thus [^{3}H]strychnine binding to a site on or near the glycine-gated chloride channel [but not identical to the glycine binding site (11)] is strongly activated by the sodium and potassium salts of several monovalent anions (12, 13), although it is inhibited by their ammonium salts (12, 14). The interaction of glycine and other agonists with the glycine binding site was also modified by

J.C.G.M. is the recipient of a Fulbright Fellowship (Spanish Ministry of Education and Science-CIES).

ABBREVIATIONS: GABA, γ-aminobutyric acid; TBPS, *t*-butylbicyclophosphorotionate.

monovalent anions (11, 15). A good correlation was found between the potency (IC_{50}) of the ammonium salts of anions to inhibit [^{3}H]strychnine binding and the permeabilities of these anions through glycine-gated chloride channels (14). Similarly, a good correlation was observed between the potencies (EC_{50}) of the sodium salts of those anions to enhance [^{3}H]strychnine binding and their relative permeabilities through glycine-gated chloride channels (16). Moreover, the increase in the apparent affinity for [^{3}H]strychnine produced by a fixed concentration (100 mM) of these anions was highly correlated with the relative permeabilities of these anions through glycine-gated chloride channels (16). These findings suggest that the effect of anions on [^{3}H]strychnine binding is directly related to the mechanisms that regulate their permeabilities through glycine-gated chloride channels.

In this latter study (16), it was observed that the sodium salts of several monovalent anions produced a significant inhibition of [^{3}H]strychnine binding after the initial phase of activation. This inhibition could not be attributed solely to high ionic strength because the maximum inhibition differed between ions. Moreover, inhibition of [^{3}H]strychnine binding below the control values was observed with some of the anions, suggesting that this inhibition phase was not simply a reversal of the activation phase. These observations suggest that anion inhibition of [^{3}H]strychnine binding could be related to a specific action at glycine-gated chloride channels. In the present report, we demonstrate that both the activation and the inhibition phases of the effect of anions on [^{3}H]strychnine binding can be described by models containing two anion binding sites. These models give direct estimates of the affinities of both anions and ligand for their respective binding sites and also provide other parameters that describe the interactions between these sites.

Experimental Procedures

Materials. [^{3}H]Strychnine sulphate (30 Ci/mmol) was obtained from Amersham (Arlington Heights, IL). Strychnine sulphate was obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals were purchased from standard commercial sources.

[^{3}H]Strychnine binding. Spinal cords and medullae oblongatae were obtained from adult male Sprague-Dawley rats (Taconic Farms, Germantown, NY). Synaptic membranes (purified P_2 fractions) were prepared as described (11) and stored at −20° until used. [^{3}H]Strychnine binding was determined as described (16). In brief, synaptic membranes were incubated with [^{3}H]strychnine in 50 mM sodium-potassium phosphate buffer (pH 7.1) at 0–4° for 20 min. The incubation was terminated by filtration using a Brandel M-24 filtering manifold. Anion concentrations were increased by addition of solutions of the corresponding sodium salt, the pH of which was adjusted to 7.1. Specific [^{3}H]strychnine binding was defined as the binding displaceable by 1 μM strychnine.

Protein determination. The Miller (17) modification of the technique of Lowry *et al.* (18), with bovine serum albumin as a standard, was used.

Two-sites models for the interaction of anions with glycine-gated chloride channels. Two different models were constructed to account for the biphasic effect of anions on [^{3}H]strychnine binding. Both models propose two binding sites for anions at glycine-gated chloride channels (Fig. 1).

The first of these models (activation-inhibition) consists of two distinct binding sites for anions and a third, separate, binding site for [^{3}H]strychnine (Fig. 1A). In this model, the biphasic effect of anions is explained by the supposition that binding of the anion to either of these sites will induce conformational changes producing an opposite effect in the ligand binding site. Hence, the binding of the anion to one site (site 1) will increase the affinity for the ligand, producing an activation, whereas the binding of the anion to the other site (site 2) will decrease the affinity for the ligand, producing the inhibition phase. The second model (equivalent sites model, meaning that the sites are kinetically equivalent in their interaction with anions) proposes two equal binding sites that can be occupied either by the anion or by the ligand (Fig. 1B). When one of the sites is occupied by the anion, the affinity of the remaining site for the ligand is increased, which explains the activation by anions. The inhibition phase results from the competitive displacement of the ligand by the anion when the concentration of anion becomes sufficiently high to occupy both binding sites.

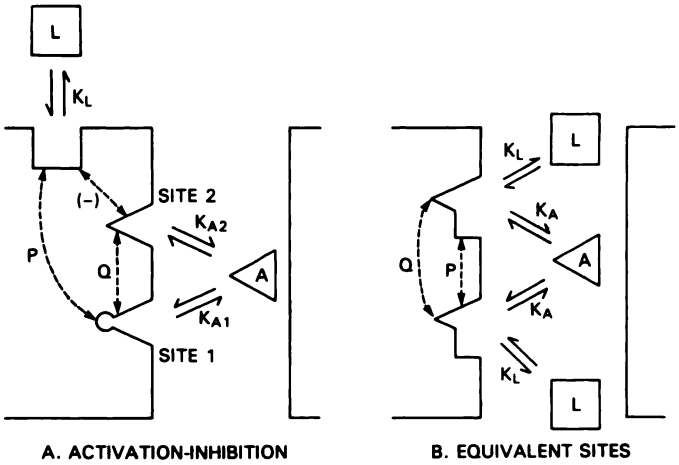
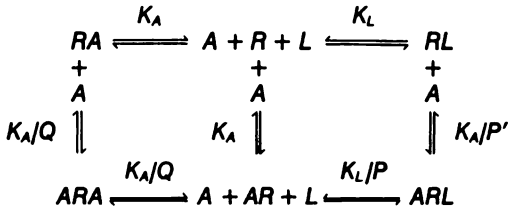


Fig. 1. Schematic diagram of models of glycine-gated chloride channels. A, Activation-inhibition model. The anion (*A*) interacts with two distinct sites at the channel. Binding to site 1 enhances (*P*) the affinity (K_L) of the ligand (*L*) for its site; binding of the anion to site 2 completely inhibits the binding of the ligand. There is a cooperative interaction (*Q*) between site 1 and site 2. B, Equivalent sites model. The ligand (*L*) and the anion (*A*) compete for two identical binding sites at the channel. Binding of the anion to one of these sites enhances (*P*) the affinity (K_L) of the ligand and modifies (*Q*) the affinity (K_A) of other molecule of anion for the remaining site.

Equations can be derived for these models where the amount of ligand bound to the receptor (*B*) is expressed as a function of the concentration of anion ([*A*]) and the concentration of ligand ([*L*]). The experimental data can be tentatively fitted to these functions to determine whether the models are able to predict the behavior of the system. In addition, the fitting procedure permits the calculation of several parameters of the function that provide useful information about the interaction of the anions with the receptor. However, even if the model is correct as far as it fits the experimental data, determining the values of its parameters may not be possible if there is a high dependency between them. High dependency occurs when certain parameters are related in the function or when there are too many variable parameters. If this happens, the function must be simplified in order to calculate meaningful parameter values. Because high dependency occurred in the original form of these models, several assumptions were made to reduce their number of variable parameters. Assumptions in the activation-inhibition model: 1) the affinity of the ligand for its binding site is markedly reduced when the inhibitory binding site is occupied by anion. In this situation, the probability of ligand binding to receptor is negligible. Also 2) the equilibrium dissociation constants of an anion for the stimulatory site (K_{A1}) and the inhibitory site (K_{A2}) are equal ($K_{A1} = K_{A2} = K_A$). Preliminary estimates fitting the data to a function that permitted different anion affinities to these sites resulted in a good fitting but high dependency between K_{A1} and K_{A2}. These preliminary results were consistent in all cases with equal values for the affinities of the anions. In the equivalent sites model, one assumption was made. Because there are two ligand binding sites per

receptor, the binding of the first molecule of ligand can affect the binding of another molecule of ligand to the remaining binding site, displaying positive cooperativity, negative cooperativity, or no cooperativity. However, saturation experiments with [^{3}H]strychnine provided no evidence of cooperativity for this ligand (Table 2; see also Refs. 11, and 13). Thus, it was assumed that no cooperativity was involved in the binding of the ligand to the receptor. Moreover, to further simplify the model, the possibility that two molecules of ligand could simultaneously bind to the receptor was excluded, a condition more restrictive than no cooperativity.

If R is the receptor, L the ligand, and A the anion, the following reactions can occur in either of these models when the assumptions formulated above are taken into account:



where K_L is the equilibrium dissociation constant for the binding of the ligand to its site when the other sites are not occupied; K_A is the equilibrium dissociation constant for the binding of the anion to one of its binding sites when the other sites are not occupied; P is a parameter that indicates how the affinity of the ligand is modified by the binding of the anion; P' indicates how the affinity of the anion is modified by the binding of the ligand; and Q indicates how the affinity of the anion for one binding site is modified by the binding of another molecule of anion to the remaining binding site (cooperativity for the anions).

The assumptions formulated above rule out the formation of complexes such as *RAL*, *ARAL*, and *LRL*. Further, the two complexes that can be formed with the receptor and one molecule of anion (*RA* and *AR*) are equivalent because $K_{A1} = K_{A2} = K_A$. Thus, the same complexes will be formed in both models, *RL*, *AR*, *ARA*, and *ARL*, and both models can be formulated with an identical equation. The equilibrium dissociation constants for the reactions of formation of these complexes will be:

$$K_L = \frac{[R]\cdot[L]}{[RL]};\quad K_A = \frac{[R]\cdot[A]}{[AR]};\quad \frac{K_L}{P} = \frac{[AR]\cdot[L]}{[ARL]};\quad \frac{K_A}{Q} = \frac{[AR]\cdot[A]}{[ARA]};\quad \frac{K_A}{P'} = \frac{[RL]\cdot[A]}{[ARL]} \qquad (1)$$

where P, P', and Q are parameters reflecting the extent to which the affinity of a site is modified when the other sites are occupied. It can be easily demonstrated from the equilibrium condition that $P' = P$.

If B is the number of binding sites occupied by ligand and B_{max}, the total number of binding sites for the ligand, the fractional occupany will be:

$$\frac{B}{B_{max}} = \frac{[RL] + [ARL]}{[R] + [RL] + [AR] + [ARA] + [ARL]} \qquad (2)$$

In saturation experiments B is determined in the presence of increasing concentrations of ligand. Substituting the Eqs. 1 in Eq. 2 and solving, B can be expressed as the following function of $[A]$ and $[L]$:

$$B([A],[L]) = \frac{B_{max}\cdot[L]}{K_L\cdot\dfrac{1 + \dfrac{[A]}{K_A} + Q\cdot\dfrac{[A]^2}{K_A^2}}{1 + P\cdot\dfrac{[A]}{K_A}} + [L]} \qquad (3)$$

Therefore, this model predicts that the number of binding sites (B_{max}) is not modified in the presence of anion, but only the apparent affinity ($K_{L_{app}}$) for the ligand, which is:

$$K_{L_{app}}([A]) = K_L\cdot\frac{1 + \dfrac{[A]}{K_A} + Q\cdot\dfrac{[A]^2}{K_A^2}}{1 + P\cdot\dfrac{[A]}{K_A}} \qquad (4)$$

B can also be expressed as a function of $[A]$ for experiments in which $[L]$ is fixed and B is studied in the presence of increasing concentrations of anion. In these experiments the amount of ligand bound in the absence of monovalent anions (B_o) is also determined. Thus, B_o can be substituted for B_{max}, which is not calculated in these experiments. In the absence of anion ($[A] = 0$) Eq. 3 becomes:

$$B_o = \frac{B_{max}\cdot[L]}{K_L + [L]}$$

Solving for B_{max} and substituting in Eqs. 2 and 1:

$$B([A]) = \frac{B_o\cdot(K_L + [L])\cdot(1 + \dfrac{P}{K_A}\cdot[A])}{(K_L + [L]) + \dfrac{K_L + P\cdot[L]}{K_A}\cdot[A] + \dfrac{Q\cdot K_L}{K_A^2}\cdot[A]^2} \qquad (5)$$

In this equation, $[L]$ becomes an additional parameter. Fig. 2 illustrates a series of curves generated from Eq. 5, examining the effects of modifying the parameters K_A, P, and Q. Changes in the affinity for the anion (K_A) shift both the activation and the inhibition phases of the curves to the left or to the right, whereas the parameters P and Q affect the extent of the activation and the inhibition phases, respectively. When $Q = 0$, only the activation phase is present. Likewise, a model supposing one binding site for anions at the channel (single-site model) predicts only activation [or only inhibition (11)] of ligand binding. In fact equations deducted for a single-site model were indentical to Eqs. 3 and 5 for the two-sites models in the particular case $Q = 0$.

Data analysis. Data were analyzed using MLAB (19) on a DEC10 computer. Nonlinear regression analysis was used to fit the data to Eqs. 3, 4, or 5. Estimates of the relevant parameters were calculated by an iterative procedure using a minimum sum of squares as the criterion for goodness of fit. Saturation experiments were analyzed using the same program, simultaneously fitting (by nonlinear regression) both total binding data to the total binding function and nonspecific binding data to a straight line that passes through the origin. This procedure estimated values for the affinity ($K_{L_{app}}$), B_{max}, and the slope of nonspecific binding.

Results

Effect of varying anion concentrations on [^{3}H]strychnine binding. The effects of six monovalent anions on [^{3}H] strychnine binding to rat spinal cord membranes (16) are shown in Fig. 3. Thiocyanate, perchlorate, and iodide had a clear biphasic effect on [^{3}H]strychnine binding, with a phase of inhibition following the initial activation. [^{3}H]Strychnine binding was totally abolished by 3 M concentrations of these anions. In the concentration range employed, the beginning of an inhibitory phase was also observed with nitrate and bromide, but not with chloride.

Because a biphasic effect on radioligand binding is predicted by models assuming the presence of two anion binding sites at glycine-gated chloride channels, but not by a single-site model (11), these data were analyzed using the two models described in Experimental Procedures. Indeed, only the effect of chloride could be fitted by a single-site model. The effect of the six anions studied, including chloride, was fitted by either the activation-inhibition model or the equivalent sites model, al-

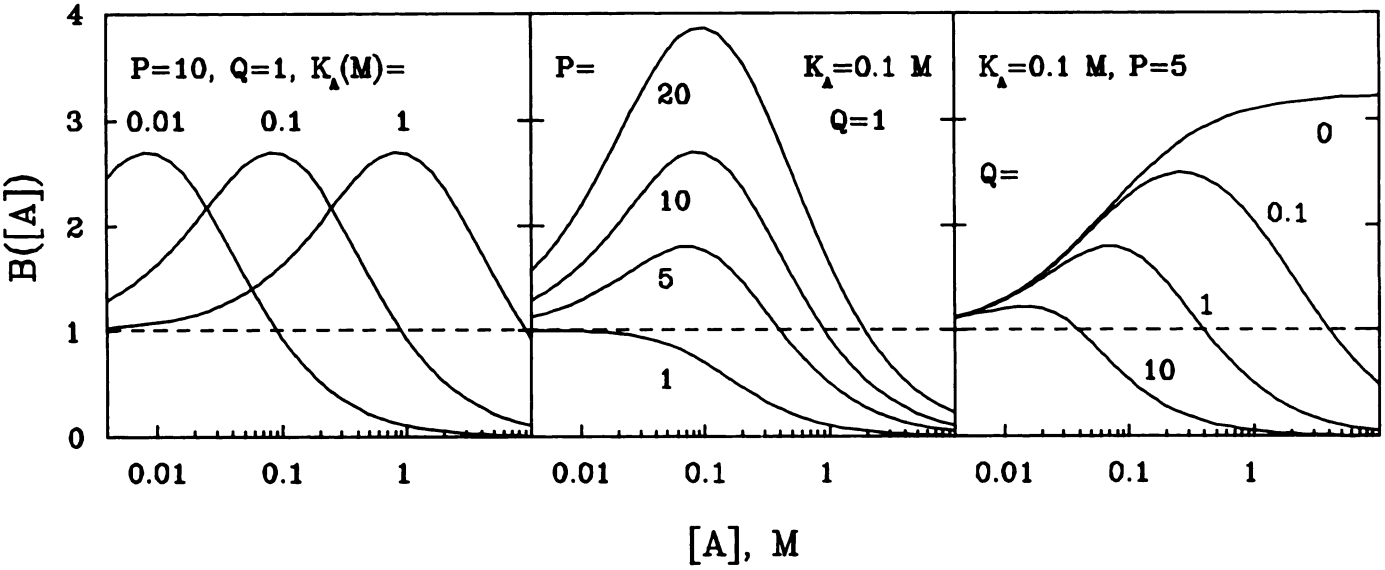


Fig. 2. Computer-generated curves for anion effect on ligand binding. Curves in the figure were generated from Eq. 5, corresponding to both the activation-inhibition model and the equivalent sites model after the assumptions indicated in the text were made. The following values were given to the parameters of Eq. 5: $B_o = 1$ (arbitrary units, the value of B_o is indicated by the *dotted line*), $[L] = 1.75$ nM, and $K_L = 11$ nM. $B[A]$ is expressed in the same units as B_o. Different values were given to the parameters K_A, P, and Q to show how they modify the shape of the curve. From *left* to *right*, the effect of changes in K_A, P, and Q were studied in each of the panels, respectively. Note that changes in K_A shift the maxima of the curves to the left or the right, whereas P and Q affect the extent of the activation and the inhibition, respectively.

though degenerate values for the parameters were obtained in the original forms of these models. Curves appearing in Fig. 3 were the result of fitting the data to Eq. 5, derived from both models after they were simplified by making several assumptions (see Experimental Procedures). Parameters B_o, $[L]$, and K_L from Eq. 5 were fixed for the fitting (Table 1), because they could be readily determined by other procedures. Thus, B_o was [^{3}H]strychnine binding in the absence of monovalent anions. The concentration of [^{3}H]strychnine in the assay ($[L]$) was also measured, and K_L was determined in saturation experiments in the absence of monovalent anions (Table 2). The other parameters (K_A, P, and Q) were determined by the fitting procedures. Despite fixing B_o, L, and K_L, the dependency between K_A, P, and Q was still high (>0.98) with some of the anions. Because a tendency of P and Q to increase together was detected, the parameter Q was further constrained to values between 0 and 1. Values of $Q > 1$ are interpreted as a positive cooperative interaction between two molecules of anion to bind to the channel, $Q < 1$ would represent negative cooperativity, $Q = 1$, no cooperativity and, at $Q = 0$, only one molecule of anion can be bound (which can be envisioned as an extreme case of negative cooperativity). When $Q = 0$ there is no inhibition phase in the effect of the anions (Fig. 2), as appears with chloride. Negative cooperativity between two anions might be predicted, because binding of two anions to the channel would result in clustering negative charges together. Hence, constraining Q to values ≤ 1 could be a realistic approximation. The goodness of the fitting with and without constraints imposed on Q was essentially the same.

Values of K_A, P, and Q for these six anions determined in experiments similar to the one illustrated in Fig. 3 are presented in Table 1. The parameter Q always reached the upper limit of the constraint ($Q = 1$) for thiocyanate, perchlorate, and iodide and was very close to 0 for chloride and nitrate. Hence, the six anions studied here appear to distribute in two groups, with high (thiocyanate, perchlorate, and iodide) and low (chloride, bromide, and nitrate) Q values, respectively.

Saturation experiments in the presence of anions. Affinity ($K_{L_{app}}$) and B_{max} values for [^{3}H]strychnine binding were measured in the presence of four concentrations of monovalent anions (Table 2). The concentrations of anions were chosen to span both the activation and the inhibition phase of their effect (Fig. 3).

The models discussed above require that the enhancement and the inhibition of [^{3}H]strychnine binding are due to an increase and a decrease, respectively, in the apparent affinity of the ligand. As it can be seen in Table 2 and Fig. 4, this condition was met by all six anions studied. By contrast, a unidirectional change in the affinity, either increase or decrease, is predicted by a single-site model (11). Only the effect of chloride was consistent with that model. Moreover, no changes in the B_{max} for [^{3}H]strychnine were observed in the presence of the anions, except when >2 M perchlorate and iodide were employed (Table 2). This decrease in B_{max} can probably account for the deviation from the theoretical curves of the points at the highest concentrations of iodide and perchlorate (Fig. 3). A similar decrease in the B_{max} at 3 M thiocyanate was probably responsible for the deviation of the corresponding point in Fig. 3, although the B_{max} at 2 M thiocyanate was not significantly lower than the B_{max} of the control (Table 2).

The values of $K_{L_{app}}$ in Table 2 can be fitted to Eq. 4 to calculate the parameters of the models without the interference of the changes in B_{max}, where present. The values of the parameters obtained this way (Fig. 4) were very similar to those shown in Table 1, except in the case of chloride, which appears to have a higher affinity and lower P value with this method. Also, the value for $K_{L_{app}}$ with 2 M perchlorate appears to be anomalous and was not included in the fitting, because [^{3}H] strychnine binding at this concentration of perchlorate is very low and likely to yield unreliable results. Indeed, taking this

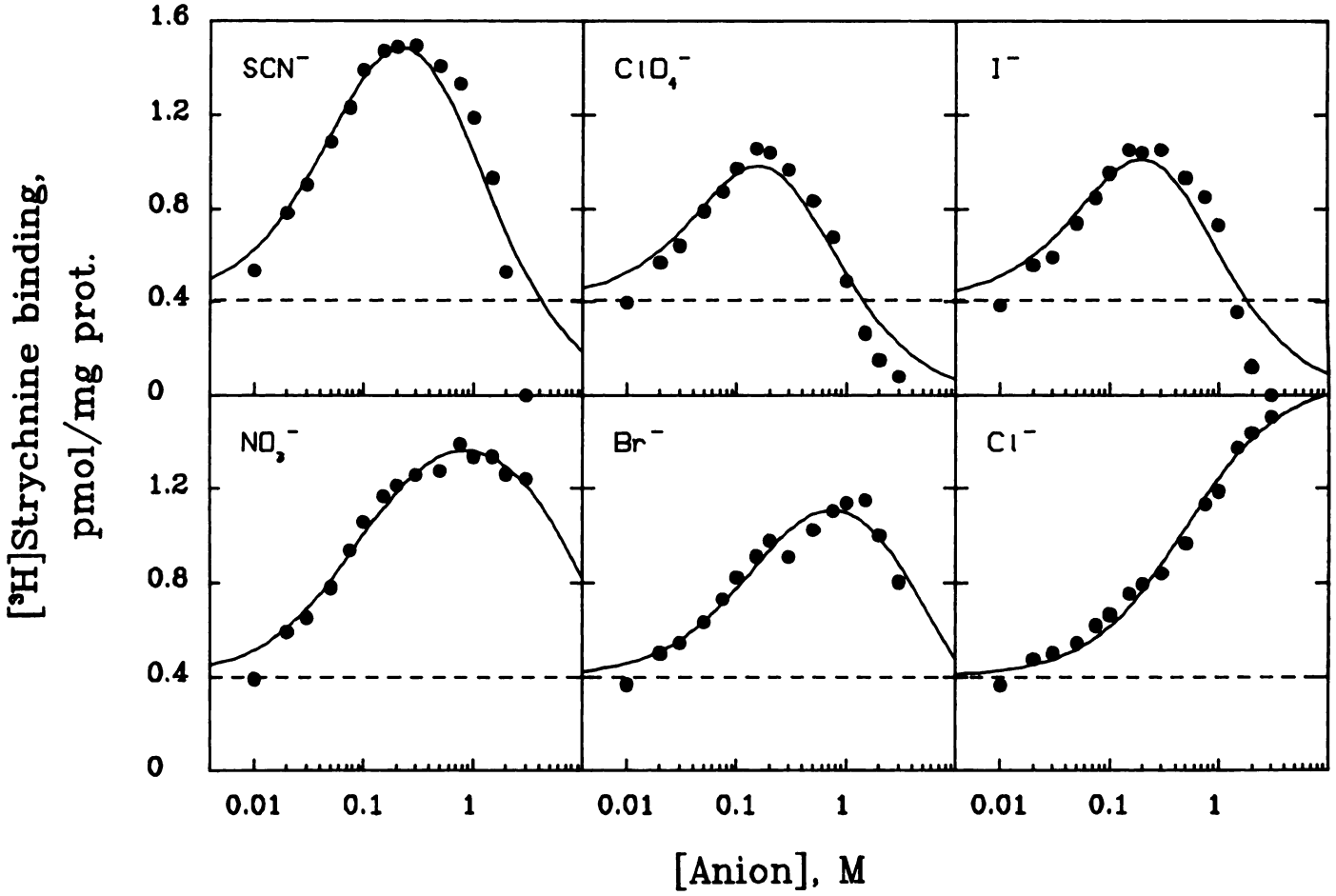


Fig. 3. Dose-response effect of anions on [^{3}H]strychnine binding. Spinal cord membranes were incubated with 1.75 nM [^{3}H]strychnine in the presence of increasing concentrations of the sodium salts of the anions indicated, as described in Experimental Procedures. *Horizontal dotted lines* indicate the value of the control in the absence of anions. Each *point* represents the mean of three determinations, with standard error of less than 5% of the indicated value. *Curves* were obtained by nonlinear regression fitting of the points to eq. 5. The parameters B_o, K_L, and [L] were fixed for the fitting to the following values: B_o = 402 fmol/mg of protein, K_L = 11 nM (Table 2), and [L] = 1.75 nM. B_o and [L] were measured in the same experiment, whereas K_L was determined in separate saturation experiments. The parameters K_A, P, and Q were allowed to vary during the fitting. Table 1 shows the means of the parameters obtained in this and two other similar experiments. The correlation coefficients obtained in the fittings were 0.86 (SCN^-), 0.93 (ClO_4^-), 0.86 (I^-), 0.98 (NO_3^-), 0.94 (Br^-), and 0.99 (Cl^-).

TABLE 1

Parameters obtained by fitting dose-response curves for anions to Eq. 5

The values of the parameters K_A, P, and Q were calculated by fitting dose-response curves for anions to Eq. 5, as explained in Experimental Procedures. The constraints used in the fitting were $K_A > 0$, $P > 1$, $0 < Q \leq 1$. Values are the mean ± standard error of three independent experiments. The values of the constant parameters B_o and [L] in these three experiments were B_o = 402, 300, and 275 fmol/mg of protein and [L] = 1.75, 1.87, and 2.02 nM, respectively. K_L was fixed to a value of 11.0 nM (Table 2) for every experiment. The data of the last column (K_m) are the apparent affinities of the anions for glycine-gated chloride channels calculated from the fitting to one-site model of electrophysiological data (7).

Anion	K_A	P	Q	K_A/P	K_m[a]
	mM			mM	mM
SCN^-	227 ± 12	26.4 ± 4.2	1.00 ± 0.00	9 ± 2	4
ClO_4^-	204 ± 32	8.1 ± 0.7	1.00 ± 0.00	26 ± 6	
I^-	244 ± 9	9.6 ± 0.4	1.00 ± 0.00	25 ± 1	25
NO_3^-	279 ± 74	11.6 ± 2.5	0.08 ± 0.04	24 ± 2	
Br^-	371 ± 36	8.7 ± 1.3	0.20 ± 0.05	44 ± 3	39
Cl^-	1416 ± 210	13.3 ± 2.5	0.00 ± 0.00	109 ± 7	111

[a] Data from Bormann *et al.* (7).

point into account resulted in low correlation coefficients (r = 0.72) and very low values for K_A (0.2 mM) and Q (0.0). Otherwise, very good correlation coefficients were obtained in these fittings, and the values presented in Table 1 were confirmed by this method.

Another way to analyze the saturation data in the presence of different concentrations of anions is fitting directly the raw data to the surface defined by Eq. 3. One experiment analyzed this way (data not shown) resulted in good correlation coefficients for nitrate (r = 0.94), thiocyanate (r = 0.88), bromide (r = 0.92), and chloride (r = 0.82). Less satisfactory fittings were obtained for perchlorate (r = 0.75) and iodide (r = 0.73), because this method can not take into account the changes in B_{max} observed at high concentrations of these anions. Parameter values calculated by this method were similar to those obtained with the other procedures.

Discussion

In this study, the biphasic effect of anions on [^{3}H]strychnine binding was analyzed using models that assume two binding sites for anions at glycine-gated chloride channels. In the

TABLE 2

Effect of anions on the affinity of the B_{max} of [^{3}H]strychnine

Saturation experiments using nine concentrations of [^{3}H]strychnine (1–50 nM) were performed in the presence of the indicated concentrations of anions (sodium salts). Control values were determined in the absence of monovalent anions. Values for $K_{L_{app}}$ (nM) and B_{max} (fmol/mg of protein) were calculated by simultaneous fitting of total and nonspecific data using nonlinear regression, as described in Experimental Procedures. Values are the mean ± standard error of three to seven independent experiments. Standard errors obtained in the fitting of the individual experiments were always smaller than the errors indicated in the table.

Anion	[Anion]	K_{L}app	B_{max}
	M	nM	fmol/mg of protein
Control		11.0 ± 2.0	4239 ± 539
ClO_4^-	0.05	5.0 ± 0.9	3781 ± 859
	0.20	3.0 ± 0.7*	3899 ± 501
	1.00	7.4 ± 2.2	3750 ± 503
	2.00	3.3 ± 0.9*	993 ± 221*
SCN^-	0.05	2.8 ± 0.7*	4106 ± 348
	0.20	1.9 ± 0.3*	4308 ± 143
	1.00	2.3 ± 0.9*	3845 ± 326
	2.00	6.0 ± 2.6	3432 ± 455
NO_3^-	0.05	4.7 ± 1.0*	3927 ± 519
	0.20	2.4 ± 0.8*	3873 ± 510
	1.00	2.0 ± 0.9*	4115 ± 451
	2.00	2.5 ± 1.1*	4234 ± 506
Cl^-	0.05	6.4 ± 1.4	3434 ± 745
	0.20	4.9 ± 1.0*	3824 ± 437
	1.00	2.6 ± 0.8*	4095 ± 412
	2.00	1.9 ± 0.9*	4236 ± 380
Br^-	0.05	5.3 ± 1.1*	3950 ± 294
	0.20	3.3 ± 0.5*	3671 ± 669
	1.00	2.5 ± 0.7*	3749 ± 405
	2.00	2.7 ± 0.8*	3782 ± 237
I^-	0.05	5.7 ± 1.4	4871 ± 579
	0.20	3.0 ± 0.6*	4187 ± 347
	1.00	5.7 ± 3.6	3330 ± 675
	2.00	9.7 ± 6.0	1038 ± 283*

* Significantly different from control ($p < 0.05$, Duncan's test).

activation-inhibition model (Fig. 1A), the two anion sites interact allosterically with the ligand binding site, one of them activating [^{3}H]strychnine binding and the other inhibiting it upon the binding of the anion. In the equivalent sites model (Fig. 1B), the two anion binding sites are equal and recognize both anion and ligands. The enhancement of ligand binding is explained in this model by an increased affinity of one site for the ligand when the other site is occupied by the anion. As the concentration of anion increases, both sites are increasingly occupied by anion molecules that competitively displace the ligand, producing the observed inhibition phase.

Both models were able to provide a good fitting of the concentration-response effect on [^{3}H]strychnine binding of the six anions studied. In addition, several parameters that characterize the interaction of each of these anions with the receptor were calculated using a function common to these two models (Eq. 5), obtained when several assumptions were made. These assumptions were necessary to simplify the models and are consistent with experimental observations (see Experimental Procedures). Very similar affinities (K_A = 200–300 mM) were found for all anions tested, with the exception of chloride, which had a significantly lower affinity (Table 1). In addition to the six anions studied in detail here, these models were also able to describe the effects of acetate and fluoride on [^{3}H] strychnine binding. Although Eq. 5 cannot provide reliable values of the parameters for acetate and fluoride, the data obtained with these two anions (16) indicate that their affinities for glycine-gated chloride channels were at least 2 or 3 times lower than the affinity of chloride. These findings are consistent with the reduced permeability of glycine-gated chloride channels for acetate and fluoride (7).

Concentrations of thiocyanate, perchlorate, and iodide in excess of 1 M consistently inhibited [^{3}H]strychnine binding to a greater extent than the values predicted by the models. This is probably due to the decrease in B_{max} detected under these conditions in addition to the changes in the affinity ($K_{L_{app}}$).

Both models predict that the enhancement and inhibition of [^{3}H]strychnine binding is due to increases and decreases, respectively, in the affinity for the receptor, with no changes in B_{max}. Saturation experiments in the presence of four different concentrations of these anions confirmed this prediction, except in the case of the highest concentrations of perchlorate and iodide, at which a significant decrease in the B_{max} was observed together with the decrease in the affinity (Table 2). The decrease in B_{max} seen at these high concentrations could be due to some nonspecific effect of these anions on the receptor, in addition to the effects explained by the models. The results shown here are consistent with previous studies on the effects of chloride (12, 20) or fixed concentrations of several anions (16) on [^{3}H]strychnine binding.

The effect of anions on the affinity for [^{3}H]strychnine can be examined by fitting to these models the data from saturation experiments in the presence of anions (Table 2). We have done this in two different ways; by fitting the $K_{L_{app}}$ values obtained to the expression predicted by the model (Eq. 4) and by fitting the raw data from saturation experiments to the surface described by Eq. 3. The parameter values obtained in the first way were very similar to those deduced from concentration-response experiments, except in the case of chloride. The data in Fig. 4 indicate that chloride may have an affinity for glycine-gated chloride channels similar to that of the other anions tested, rather than the apparently higher values obtained in concentration-response experiments. Such differences may be due to the fact that the enhancement produced by chloride may not have reached its maximum at the highest concentration employed (3 M), which would result in inaccuracies in estimating parameters for this anion from concentration-response data.

Whereas the affinities of glycine-gated chloride channels for the anions (K_A) calculated in this study are different from the values obtained in electrophysiological experiments (7), in those experiments the affinities for chloride and thiocyanate deduced from conductance-activity relations also differed by 1 order of magnitude from the affinities calculated by fitting current-voltage relations to a single-site model (7). These latter findings were interpreted as indicating the presence of multiple anion binding sites in the channel, but new affinity values from a two-site model could not be obtained. In the present study, dividing K_A by the parameter P results in values very similar to those of K_m obtained by Bormann *et al.* (7) from their single-site model (see Table 1). In our models, K_A/P represents the affinity of the anion for its binding site when the ligand (strychnine) is bound to the receptor, whereas Bormann *et al.* obtained their data in the presence of glycine. This might indicate that strychnine must bind to the open conformation of the channel. The electrophysiological evidence (7) suggesting the presence of two anion binding sites at glycine-gated chloride channels is supported by the finding of clusters of positively charged amino acid residues in the primary amino acid sequence of the strychnine binding subunit of the glycine receptor (21).

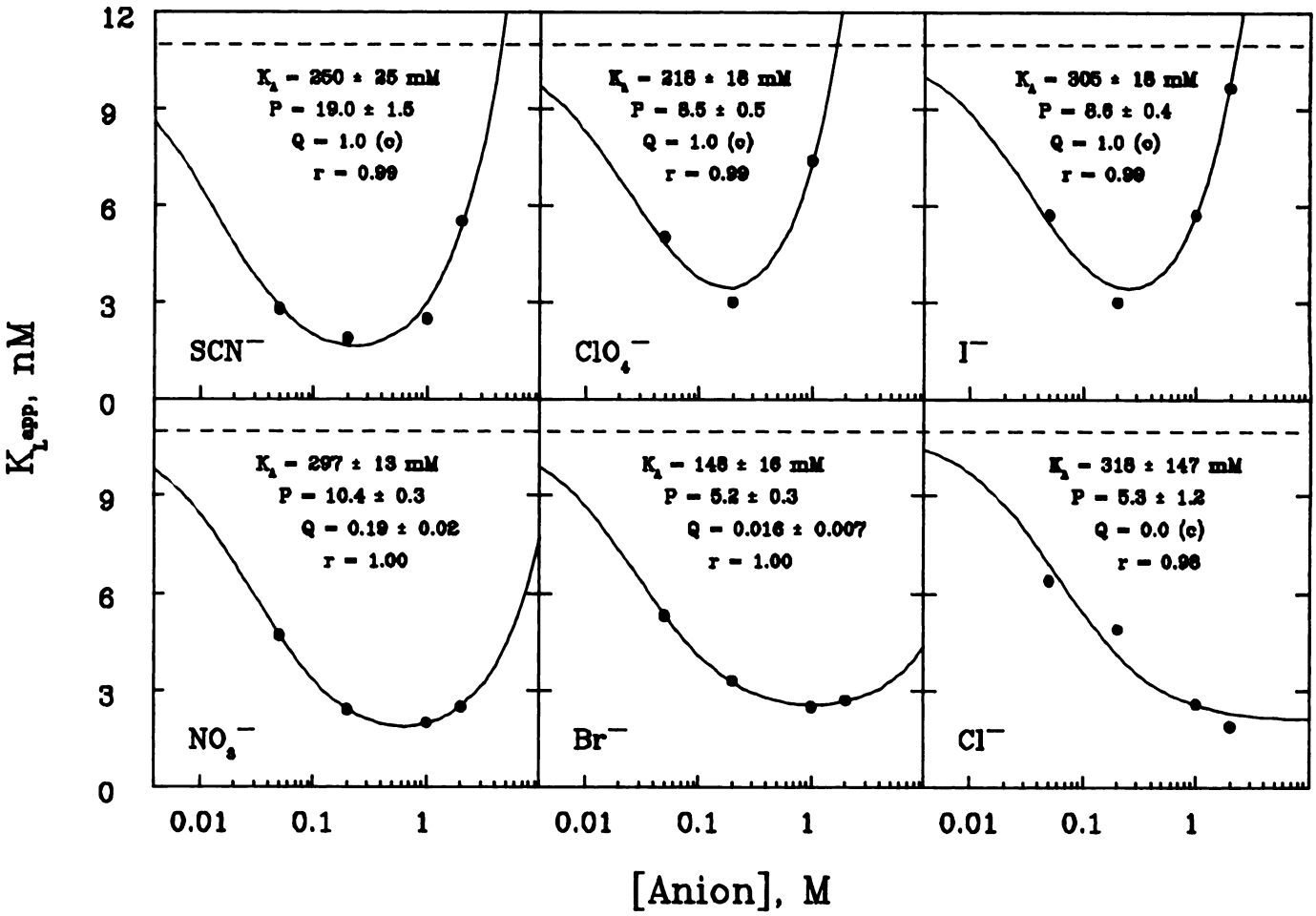


Fig. 4. Effect of anions on the affinity of [^{3}H]strychnine binding. *Curves* resulting from the fitting of $K_{L_{app}}$ values from Table 2 to eq. 4 are shown in the figure. The value of $K_{L_{app}}$ corresponding to 2 M perchlorate was not included in the fitting. Values of the parameters K_A, P, and Q obtained are shown in each of the panels, together with the correlation coefficients for the fitting (r). A value of Q followed by (c) indicates that the value for this parameter was maintained constant throughout the fitting in that particular case. The value of K_L, indicated by the *dotted lines*, was determined in the absence of monovalent anions and used as a constant parameter in eq. 4. K_L did not change significantly if it was considered a free parameter for the fitting.

These positively charged residues seem to group at both the extracellular and the intracellular mouth of the channel and may form two anion binding sites that provide anion selectivity.

The parameter Q provides an estimate of the cooperativity between two anions binding simultaneously to the channel. When the values obtained for the parameter Q are considered, the six anions examined cluster in two groups. Thiocyanate, perchlorate, and iodide had values of $Q \geq 1$, which means that two anion molecules can bind to the channel. Conversely, nitrate, bromide, and chloride had Q values that approach 0, which indicates that the simultaneous binding of two anion molecules to the channel may be hindered. These anions appear to cluster in the same two groups in a variety of experiments. For example, muscimol-stimulated $^{36}Cl^-$ uptake into rat brain synaptoneurosomes was equally and potently enhanced in the presence of bromide, chloride, and nitrate, whereas iodide and thiocyanate were unable to increase basal $^{36}Cl^-$ uptake (22). Although these effects were mediated by GABA-gated chloride channels, there is increasing evidence that glycine- and GABA-gated chloride channels share many properties, especially in regard to their interaction with anions (7, 16) and the high degree of homology observed between the α- and β-subunits of the GABA receptor complex and the [^{3}H]strychnine binding site (21, 23). Chloride and bromide also have higher conductances through glycine-gated chloride channels than iodide and thiocyanate. Indeed, good rank-order correlations were found between the values of Q for chloride, bromide, iodide, and thiocyanate and both the conductances ($r = 0.95$) and permeabilities ($r = 0.85$) of these anions through glycine-gated chloride channels (electrophysiological data from Ref. 7). These findings suggest that the ability of an anion to occupy two binding sites at the channel (high Q values) is directly related to its permeability through the channel and inversely related to its conductance, explaining why the permeability sequence and the conductance sequence for monovalent anions are in inverse order. It is possible that in the case of anions with high Q values (e.g., thiocyanate) two anion molecules can occupy the lumen of the channel at the same time in a stable conformation that causes a decreased conductance. Conversely, in cases in which low Q values are obtained (e.g., chloride), the binding of a second molecule of anion to the channel removes ("pushes") the first anion away from the channel (and into the cell), increasing the conductance but decreasing the permeability because fewer ions are able to bind to the channel.

Because the two models proposed were consistent with the data, both appear to be equally valid in this experimental setting. The principal difference between these models concerns the way they explain the inhibition of [^{3}H]strychnine binding by anions, allosteric inhibition in the case of the activation-inhibition model and competitive inhibition in the case of the equivalent sites model. Allosteric interactions at the glycine receptor, such as inhibition by glycine of [^{3}H]strychnine binding, have been evinced using protein-modifying reagents like diazonium tetrazole and acetic anhydride (11, 13). In experiments pretreating membranes with acetic anhydride, the enhancement of [^{3}H]strychnine binding by anions was abolished, but the inhibitory effect of thiocyanate and perchlorate was unaffected (data not shown). Therefore, acetylation of certain amino acid residues appears to disrupt the allosteric interaction responsible for the activation of [^{3}H]strychnine binding. Furthermore, these results suggest that the inhibition by anions may be competitive, as proposed by the equivalent sites model, because it was not affected by acetylation. However, the requirement in the equivalent sites model that strychnine binds to the same sites as anions, although suggested by several observations (11, 12, 14, 16, 20), is not conclusive yet.

These models might also be useful to describe the interaction of anions with GABA-gated chloride channels, because the effect of anions on [^{3}H]strychnine binding to the glycine receptor remarkably resembles their effects on [^{35}S]TBPS binding to the GABA receptor (16, 24). Moreover, these models may prove useful in the analysis of other cases of biphasic effects on radioligand binding, i.e., activation followed by inhibition.

In summary, models based on the presence of two anion binding sites at glycine-gated chloride channels are able to explain the biphasic effect of anions on [^{3}H]strychnine binding in either dose-response or saturation experiments, whereas a single-site model can only explain the effect of chloride. A different experimental approach will be needed to distinguish between the possibilities suggested by each of these models, that is, whether these anion binding sites are equivalent and whether strychnine acts at this or at a distinct site.

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