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Picrate and Niflumate Block Anion Modulation of Radioligand Binding to the γ-Aminobutyric Acid/Benzodiazepine Receptor Complex

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SUMMARY

The organic anions picrate (2,4,6-trinitrophenol) and niflumate (2-[[3-(trifluoromethyl)phenyl]-amino]-3-pyridinecarboxylate) were examined for their effects on radioligand binding to the γ-aminobutyric acid (GABA)/benzodiazepine receptor complex. Neither organic anion produced the enhancement of [^{35}S]*t*-butylbicyclophosphorothionate (TBPS) binding characteristic of anions (such as Cl^- and Br^-) known to permeate GABA-gated chloride channels. However, both picrate and niflumate potently (IC_{50} values between 66 and 531 and 30 and 155 μM, respectively) inhibited the effect of 100–200 mM concentrations of anions (I^-, Br^-, Cl^-, SCN^-, and F^-) to increase the binding of [^{35}S]TBPS to GABA-gated chloride channels. This inhibition resulted from a decrease in both the maximum number of binding sites and the apparent affinity (increased K_d) of [^{35}S]TBPS. Niflumate was consistently more potent than picrate, but both organic anions exhibited the same sequence of relative potencies against smaller anions ($I^- > Br^- > Cl^- > SCN^- > F^-$). This sequence was similar to that described for the relative permeabilities of these anions through GABA-gated chloride channels. Niflumate and picrate were potent inhibitors of Cl^-, but not GABA-modulated radioligand binding to benzodiazepine receptors. These findings suggest that picrate and niflumate bind with high affinity at or near an anion binding site that may regulate the movement of anions through GABA-gated chloride channels and radioligand binding at this "supramolecular complex."

Benzodiazepine receptor ligands produce their distinctive neuropharmacological effects by modulating the activity of GABA-gated chloride channels (1, 2). A functional association between benzodiazepine receptors and GABA-gated chloride channels was first evinced by Costa *et al.* (3), who showed that permeant anions such as Cl^-, Br^-, and I^- increased the apparent affinity of [^{3}H]diazepam for benzodiazepine receptors. Subsequently, permeant anions have been shown to modulate the binding characteristics of many ligands (e.g., GABA, barbiturates, pyrazolopyridines, "cage" convulsants, and β-carbolines) at this receptor/anion channel complex ("supramolecular complex") (4–9).

Electrophysiological studies (10–13) have provided evidence for the presence of anion binding sites within GABA-gated chloride channels that impart selectivity characteristics to these channels, thereby determining the type of anions gated when ligands occupy the multiple, allosteric sites comprising the receptor portion of this complex. Conversely, neurochemical studies suggest that specific anion recognition sites also determine the ability of anions to modulate ligand binding to the supramolecular complex (14–17).

A number of molecules, including picric acid (2,4,6-trinitrophenol) and niflumic acid (2-[[3-(trifluoromethyl)-phenyl]-amino]-3-pyridinecarboxylic acid) have been shown to block inward hyperpolarizing currents carried by Cl^- in crayfish muscle (18, 19). In addition, Luu *et al.* (20) recently demonstrated that picrate inhibited muscimol-stimulated $^{36}Cl^-$ uptake into rat cortical synaptoneurosomes. These effects suggest that organic anions such as picrate and niflumate could interfere with the binding of Cl^- to its recognition site at GABA-gated chloride channels. Demonstration of this would provide an important tool to examine the relationship between the anion binding site and other components of the supramolecular complex. We now provide evidence that, at low concentrations, these organic anions act at or near an anion binding site in GABA-gated chloride channels and that this site appears to be intimately involved in the regulation of ligand binding to the GABA/benzodiazepine receptor/chloride channel complex.

Methods

Animals. Male Sprague-Dawley rats (Taconic Farms, Germantown, NY) (250–350 g) were housed in a vivarium with a 12-hr light/dark

ABBREVIATIONS: GABA, γ-aminobutyric acid; TBPS, *t*-butylbicyclophosphorothionate; DMCM, methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate; FNZ, flunitrazepam.

cycle (lights on at 0700). Rat chow and water were available *ad libitum*. Experiments were initiated between 0800 and 0930. Animals were sacrificed by decapitation, and their brains were immediately removed and placed into ice-cold 50 mM Tris-citrate buffer (pH 7.4). The brains were dissected, weighed, and disrupted in 50 volumes of buffer with a Brinkman Polytron (setting 5–6, 15 sec). Homogenates were centrifuged at 20,000 × *g* for 20 min at 4°. Tissues were resuspended and recentrifuged ("washed") five more times before final resuspension in 50 volumes of Tris-citrate buffer.

Radioligand binding assays. [^{35}S]TBPS binding was assayed by a modification of the method of Squires *et al.* (8). Incubations consisted of 0.3 ml of fresh tissue suspension (250–350 μg of protein), 0.1 ml of [^{35}S]TBPS (60–120 Ci/mmol) diluted in Tris-citrate buffer to a final concentration of 2–5 nM, and 0.1 ml of salt solutions, drugs, and/or Tris-citrate buffer to a final volume of 0.5 ml. In some experiments, saturation isotherms were constructed by diluting [^{35}S]TBPS with unlabeled TBPS 1:25, followed by serial dilution of the resulting mixture to concentrations between 0.1 and 4.0 μM. Incubations were initiated by addition of radioligand and were terminated after 2 hr (at 25°) by rapid filtration through Whatman GF/B glass fiber filters and washing with two 5-ml aliquots of assay buffer using a Brandel M-48R filtering manifold (Brandel Instruments, Gaithersburg, MD). Nonspecific binding was defined using 200 μM picrotoxinin and typically represented less than 10% of total binding at radioligand concentrations between 2 and 5 nM.

[^{3}H]DMCM binding was assayed using a modification (16) of the method of Braestrup *et al.* (9). In brief, incubations consisted of 0.2 ml of fresh tissue suspension (0.15–0.25 mg of protein), 0.1 ml of [^{3}H]DMCM (80.7 Ci/mmol, diluted in Tris-citrate buffer to a final concentration of ~1 nM), and 0.1 ml of salt solutions, drugs, and/or Tris-citrate buffer to a final volume of 1.0 ml. Incubations (0–4°) were initiated by addition of radioligand and terminated after 60 min by filtration through GF/B filters and washing with three 5-ml aliquots of ice-cold assay buffer. Nonspecific binding was defined using 10 μM FNZ and represented ~30% of total binding in the absence of permeant anions. [^{3}H]FNZ binding (90 Ci/mmol) was assayed under identical conditions, except that 0.1 ml of tissue suspension (0.075–0.125 mg of protein) was utilized. Nonspecific binding (defined using 10 μM FNZ) was usually <2% of total binding. Picric and niflumic acids were dissolved in distilled water and titrated with 1 N NaOH to produce their sodium salts. Other anions were also added as their sodium salts. All assays contained citrate anion (160–200 mM). Protein content was determined by the Miller (21) modification of the method of Lowry *et al* (22) using bovine serum albumin as a standard. The radioactivity retained by the filters was measured using a Beckman LS 5801 liquid scintillation counter. K_d and B_{max} values were estimated using both linear and nonlinear fitting methods as indicated (Lundon Software, Cleveland, OH, and MLAB, National Institutes of Health. EC_{50}, E_{max}, IC_{50}, and I_{max} values were calculated by fitting concentration response curves to the sigmoidal function $f(x)=A + (B - A)/[1 + ((10^X)^D/(10^C)^D)]$ where $f(x)$ is the binding at a concentration x of drug or anion, A is the minimum binding, B is the maximum binding, C is that concentration of drug or anion producing a half-maximum effect (EC_{50} or IC_{50}) and D is the slope function (equivalent to a pseudo-Hill coefficient) (GraphPad, ISI Software).

Materials. Picric acid was obtained from Eastman Chemicals (Rochester, NY). Niflumic acid, picrotoxinin, GABA, flurosemide, and *p*-coumaric acid were obtained from Sigma Chemicals (St. Louis, MO). α-Cyano-, *m*-, and *p*-chlorocinnamic acids were obtained from Fairfield Chemical Co. (Blythewood, SC). Anthracene-5-carboxylic, *p*- and *m*-nitrobenzoic, *p*- and *m*-aminobenzoic, and *p*-toluic acids were obtained from Aldrich Chemicals (Milwaukee, WI). Radioligands were purchased from Dupont-NEN (Boston, MA). Flunitrazepam was a gift of Hoffman-LaRoche (Nutley, NJ).

Results

Effects of picrate and niflumate on [^{35}S]TBPS binding. The effect of picrate and niflumate on [^{35}S]TBPS binding was examined in both the presence and absence of anions permeable through GABA-gated chloride channels. In agreement with previous findings (8), [^{35}S]TBPS binding was highly dependent on the presence of such anions. In the presence of citrate, a large, relatively impermeant anion, specific binding at 4.2 nM [^{35}S]TBPS binding was only ~3–4 fmol/mg of protein (Fig. 1). Both picrate and niflumate exerted biphasic effects on [^{35}S]TBPS binding in the absence of additional permeant anion. At low concentrations (1–50 μM), both produced a small increase (<20%), whereas higher concentrations (0.1–10 mM) inhibited [^{35}S]TBPS binding (Fig. 1 and data not shown).

[^{35}S]TBPS binding was increased 10–60-fold by a number of permeant anions (legend to Table 1). Both picrate and niflumate inhibited anion-stimulated increases in [^{35}S]TBPS binding (Fig. 2; Table 1), with niflumate (IC_{50}, 30–155 μM) consistently more potent than picrate (IC_{50}, 66–531 μM). The rank order of potency of a series of anions was identical ($I^- > Br^- > Cl^- > SCN^- > F^-$) at concentrations between 50 and 500 mM against both picrate and niflumate (Table 1 and data not shown). Hill slopes for picrate and niflumate inhibition of anion-stimulated [^{35}S]TBPS binding were at or slightly above unity (1.0–1.5) except for F^-. In the presence of F^-, both picrate and niflumate exhibited biphasic effects, eliciting a small (~20%) potentiation at relatively low anion concentrations and inhibiting at higher concentrations (Fig. 2). Hill slopes for inhibition of F^- (100 mM)-stimulated [^{35}S]TBPS binding were 2.4 and 1.6 for niflumate and picrate, respectively.

In order to examine whether these effects were shared by other organic anions, 11 other substances were examined for their abilities to inhibit [^{35}S]TBPS binding in the presence of 200 mM NaCl. The following compounds exhibited no activity at concentrations (of their sodium salts) of up to 5 mM: furosemide, *p*- and *m*-chlorocinnamic acids, α-cyanocinnamic acid, anthracene-5-carboxylic acid, *p*-coumaric acid, *p*- and *m*-nitrobenzoic acids, *p*- and *m*-aminobenzoic acids, and *p*-toluic acids (data not shown).

[^{35}S]TBPS binding was also examined in the presence of varying concentrations (5–5000 mM) of I^-, Br^-, and Cl^- in combination with a fixed concentration of niflumate (100 μM) or picrate (250 μM) (Table 2). Both picrate and niflumate

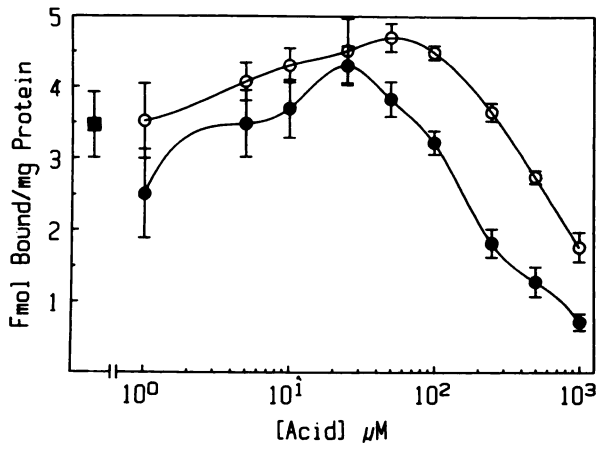

Fig. 1. Effect of niflumate (●) and picrate (○) concentration on the binding of [^{35}S]TBPS to rat cerebral cortex in the absence of permeant anion. Values represent the means ± standard errors (three experiments). One-way ANOVA indicates significant ($p < 0.01$) effect of picrate and niflumate concentration on [^{35}S]TBPS binding. The concentration of [^{35}S]TBPS was 4.2 nM.

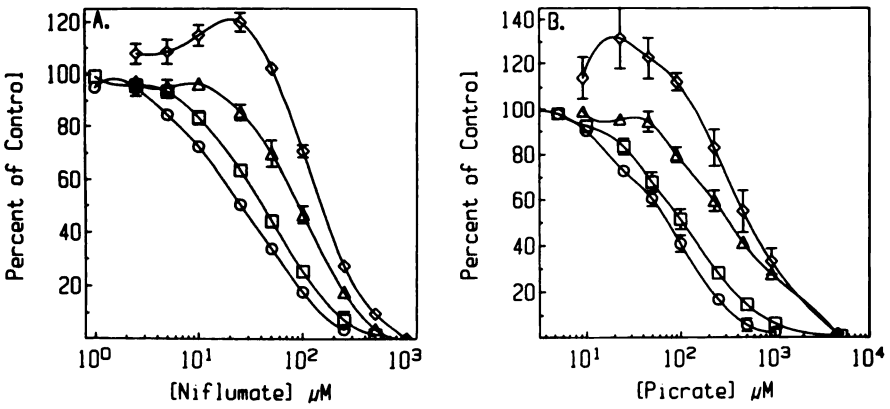

Fig. 2. Effect of niflumate (A) and picrate (B) concentration on the binding of [^{35}S]TBPS to rat cerebral cortex in the presence of 100 mM I^- (○), Br^- (□), Cl^- (△), or F^- (◇). Values are reported as percentage of control and represent the means ± standard errors (three experiments). For control values, see legend to Table 1. The concentration of [^{35}S]TBPS was 2.4 nM.

TABLE 1

Potency of niflumate and picrate versus anion enhancement of [^{35}S]TBPS binding

Values represent the means ± standard errors. Control values (in the absence of niflumate or picrate) were: I^-, 100 mM—62 fmol/mg protein, 200 mM—84 fmol; Br^-, 100 mM—150 fmol, 200 mM—179 fmol; Cl^-, 100 mM—139 fmol, 200 mM—206 fmol; SCN^-, 265 fmol; F^-, 36 fmol. The concentration of [^{35}S]TBPS was 2.4 nM.

Anion	Concentration	Niflumate IC_{50}	Picrate IC_{50}	n^a
	mM	μM	μM	
I^-	100	30 ± 3	66 ± 5	3
	200	37 ± 1	74 ± 6	5
Br^-	100	44 ± 1	101 ± 7	3
	200	64 ± 8	105 ± 18	5
Cl^-	100	97 ± 6	339 ± 35	3
	200	87 ± 3	242 ± 6	5
SCN^-	100	113 ± 4	295 ± 21	3
F^-	100	155 ± 37	531 ± 122	3

[a] Number of experiments.

TABLE 2

Effect of picrate and niflumate on halide potency and efficacy to stimulate [^{35}S]TBPS binding

Anion EC_{50} (mM) and E_{max} (fmol/mg) data were calculated as described in Methods and are expressed as means ± standard error (three experiments). The concentration of [^{35}S]TBPS was 3.6 nM.

Anion		Control	Picrate (250 μM)	Niflumate (100 μM)
I^-	EC_{50}	72.4 ± 12.7	243.1 ± 57.7[a]	116.1 ± 7.5[a]
	E_{max}	199.7 ± 30.7	133.5 ± 30.8[a]	67.8 ± 4.7[a]
Br^-	EC_{50}	230.1 ± 16.7	464.8 ± 38.5[a]	449.5 ± 46.1[a]
	E_{max}	909.5 ± 130.4	883.8 ± 57.7	426.7 ± 30.8[a]
Cl^-	EC_{50}	260.8 ± 27.1	572.9 ± 52.0[a]	751.3 ± 25.4[a]
	E_{max}	783.3 ± 100.2	>1000	>1000

[a] $p < 0.05$ compared with control (paired t test).

produced significant decreases in the potencies of these anions, with a less pronounced and consistent effect on the maximum enhancement (E_{max}). Thus, picrate and niflumate decreased the E_{max} of I^- by 53 and 66%, respectively, and niflumate reduced the E_{max} of Br^- by 53%. In contrast, both organic anions appeared to elicit a small increase in the E_{max} of Cl^--enhanced [^{35}S]TBPS binding. However, an accurate determination of the E_{max} for Cl^- was not possible in the presence of these concentrations of picrate or niflumate because [^{35}S]TBPS binding had not reached a plateau at the highest concentrations of Cl^- employed.

The effects of niflumate (100 μM) and picrate (250 μM) on the equilibrium binding parameters of [^{35}S]TBPS were also assessed in the presence and absence of 200 mM Cl^-see Fig. 3 for typical results and Table 3 for pooled data). Neither organic

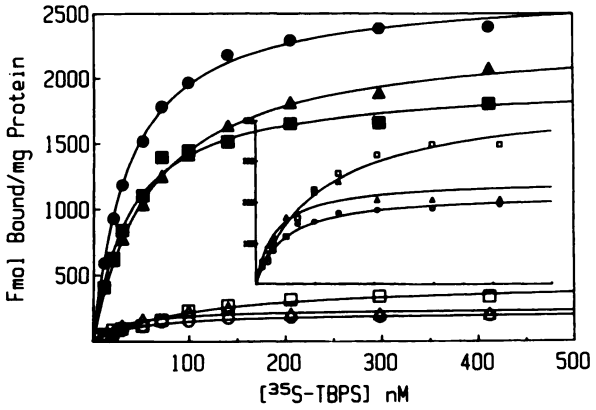

Fig. 3. Effect of 100 μM niflumate (△), 250 μM picrate (□), or neither (○) on [^{35}S]TBPS binding in the presence (*filled symbols*) or absence (*open symbols* and *inset*) of 200 mM Cl^-. Binding to rat cortical membranes was assessed in the presence of varying concentrations of [^{35}S]TBPS as described in Methods. Values are taken from a representative experiment, which was repeated twice. See Table 3 for K_d and B_{max} data.

TABLE 3

Effects of picrate and niflumate on [^{35}S]TBPS binding

[^{35}S]TBPS assayed as described in Methods, three experiments.

	No Anion		200 mM Cl^-	
	K_d	B_{max}	K_d	B_{max}
	nM	fmol/mg	nM	fmol/mg
Control	68.7 ± 18.4	460 ± 116	36.9 ± 2.6	2713 ± 156[a]
Picrate (250 μM)	115.7 ± 14.1	685 ± 129	49.2 ± 4.0[b]	1901 ± 142[b]
Niflumate (100 μM)	53.8 ± 21.1	352 ± 87	58.6 ± 6.5[b]	2108 ± 270[b]

[a] $p < 0.05$ compared to control without chloride. Two-way ANOVA indicates a significant ($p < .05$) effect of picrate vs. K_d and B_{max}, niflumate vs. B_{max}, NaCl vs B_{max}.

anion produced a significant effect on [^{35}S]TBPS binding in the absence of Cl^-, although a trend toward an increased K_d and B_{max} of [^{35}S]TBPS was observed with picrate. The B_{max} of [^{35}S]TBPS was increased 600% and the K_d was reduced ~50% in the presence of 200 mM Cl^- (Table 3; Fig. 3). Picrate- and niflumate-mediated reduction of [^{35}S]TBPS binding in the presence of Cl^- was manifest as a decrease in both apparent affinity and B_{max} of this radioligand.

Effects of niflumate and picrate on radioligand binding to benzodiazepine receptors. The effects of picrate and niflumate on radioligand binding to benzodiazepine receptors were studied in the absence and presence of permeant anions.

As previously reported (3, 9, 15, 16) both [^{3}H]DMCM and [^{3}H]FNZ binding were increased by Cl^- in a concentration-dependent fashion (Fig. 4A and data not shown). Neither picrate nor niflumate significantly affected [^{3}H]DMCM or [^{3}H]FNZ binding in the absence of permeant anion. Basal [^{3}H]DMCM binding was 206, 192, and 181 fmol/mg, and [^{3}H]FNZ binding was 823, 826, and 838 fmol/mg of protein for control, niflumate (100 μM), and picrate (250 μM), respectively (see also legend to Fig. 4). However, both organic anions inhibited the stimulatory effect of Cl^-. The inhibitory effect on [^{3}H]DMCM binding was apparent at all Cl^- concentrations (5–500 mM) but for [^{3}H]FNZ binding was readily detectable only at the highest (500 mM) Cl^- concentration. As was the case for [^{35}S]TBPS binding, niflumate was more potent than picrate (niflumate IC_{50} values are 26 and 78 μM, picrate IC_{50} values are 117 and 234 μM versus [^{3}H]DMCM and [^{3}H]FNZ, respectively, in the presence of 500 mM NaCl; Fig. 4B and data not shown).

In contrast, niflumate did not alter the potency of GABA to enhance [^{3}H]FNZ binding, whereas picrate decreased GABA potency only at very high concentrations (5 mM) (see Fig. 5B for typical results and Table 4 for pooled data). [^{3}H]DMCM assays were carried out in the presence of 500 mM Cl^- because the inhibitory effect of GABA could not be measured reliably in the absence of anion. Under these conditions, both picrate and niflumate decreased [^{3}H]DMCM binding in the absence of GABA by 60% or more (see legend to Fig. 5A). A high concentration of picrate (5 mM) increased the potency of GABA to inhibit [^{3}H]DMCM binding; lower concentrations (100–250 μM) were without significant effect. In contrast to the effect of 5 mM picrate, niflumate (2.5 mM) tended to reduce the potency of GABA (Table 4). Both anions appeared to decrease modestly the maximal inhibition by GABA, although only the effect of 2.5 mM niflumate reached statistical significance.

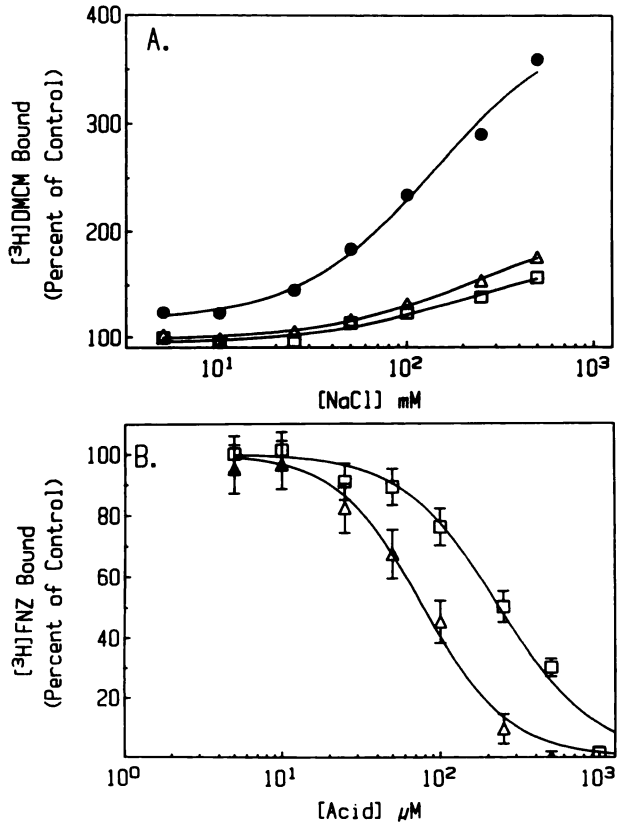

Fig. 4. Effect of 100 μM niflumate (△), 250 μM picrate (□), or neither (●) on binding of [^{3}H]DMCM at indicated Cl^- concentrations (A) and indicated concentrations of niflumate (△) or picrate (□) on binding of [^{3}H]FNZ at 500 mM Cl^- (B). Values are reported as percentage of control (control binding: A, 206, 192, and 181 fmol/mg for control, niflumate, and picrate, respectively; B, 823 fmol/mg of protein). Radioligand concentrations were 1.1 (A) and 1.9 nM (B). ANOVA indicates significant $p < 0.001$ effect for picrate and niflumate.

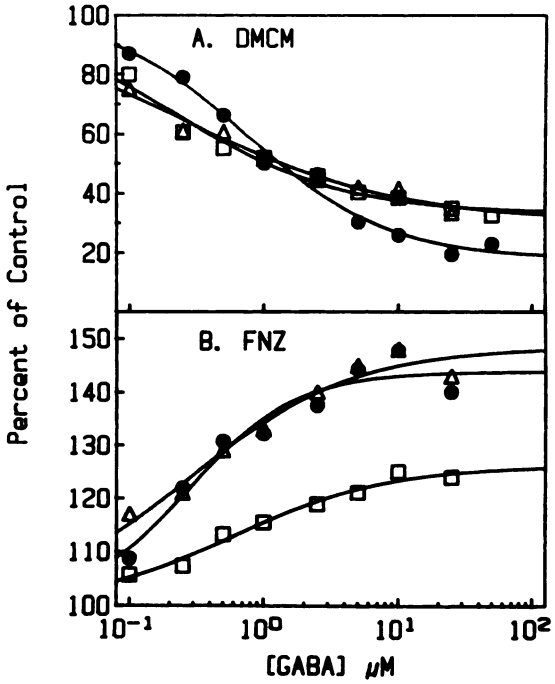

Fig. 5. Effect of 100 μM niflumate (△), 250 μM picrate (□), or neither (●) on binding of [^{3}H]DMCM (A) and [^{3}H]FNZ (B) to rat cortical membranes at indicated concentrations of GABA and, for [^{3}H]DMCM, in the presence of 500 mM Cl^-. Values are reported as percent of control and taken from a representative experiment, which was repeated twice (control binding: A, 229, 85, 80, and B, 648, 622, and 628 fmol/mg of protein for control, niflumate, and picrate, respectively). Radioligand concentrations were 1.3 (A) and 1.4 nM (B).

TABLE 4

Effect of picrate and niflumate on gabaergic modulation of benzodiazepine agonist and inverse agonist binding

GABA IC_{50}, I_{max}, EC_{50}, and E_{max} data were calculated from data illustrated in Fig. as described in Methods and are expressed as means ± standard errors (three experiments for all groups except controls, five experiments).

	[^{3}H]DMCM		[^{3}H]FNZ	
	GABA IC_{50}	GABA I_{max}	GABA EC_{50}	GABA E_{max}
	nM	%	nM	%
Control	694 ± 71	87.7 ± 5.2	301 ± 17	143.7 ± 4.8
Picrate				
250 μM	351 ± 144	64.7 ± 3.3	393 ± 68	126.1 ± 1.6*
5 mM	220 ± 70*	59.0 ± 13.2	1508 ± 159*	121.2 ± 2.3*
Niflumate				
100 μM	451 ± 119	66.6 ± 3.3	389 ± 110	152.8 ± 3.2
2.5 mM	1800 ± 1210	51.7 ± 3.7*	309 ± 133	132.7 ± 2.8

* $p < 0.05$ compared with control (paired t test).

Discussion

The effector component of the GABA/benzodiazepine receptor complex is an ionophore, selectively permeable to small, negatively charged anions such as Cl^-. Electrophysiological studies indicate these channels do not act as simple water-filled

pores but exhibit behavior suggesting the presence of a selectivity filter or anion binding site within the ionophore (10–13). The permeability of anions through GABA-gated chloride channels is highly correlated with their potencies to enhance the binding of the "cage" convulsant [^{35}S]TBPS, as well as both agonist and inverse agonists, at benzodiazepine receptors (15–17). These observations strongly suggest that occupation of anion binding sites modulates radioligand binding to the supramolecular complex. This relationship, coupled with the ability of the organic anions picrate and niflumate to block Cl^- currents in electrophysiological studies (18, 19), led us to investigate their effects on radioligand binding to the GABA/benzodiazepine receptor complex.

Neither picrate nor niflumate displayed behavior typical of small anions on [^{35}S]TBPS binding. Whereas halides produced a robust, concentration-dependent increase in [^{35}S]TBPS binding (Table 2) (8, 15), neither picrate nor niflumate was efficacious in stimulating [^{35}S]TBPS binding, even at concentrations approaching their solubility limits (~10 mM). However, both picrate and niflumate potently *inhibited* the effect of halides, with IC_{50} values estimated in the low micromolar range (against millimolar concentrations of anions). The relatively large size of picrate and niflumate compared with chloride or bromide (even in their hydrated states) suggests that these organic anions are unable to pass through the channel component of the supramolecular complex but may hinder access to the putative anion-binding site by smaller, more permeant anions. This hypothesis is supported by several lines of evidence.

Although niflumate more potently inhibited anion-supported [^{35}S]TBPS binding than did picrate, both exhibited the same relative potencies against the anions tested ($I^- > Br^- > Cl^- > SCN^- > F^-$; Table 1). With the exception of SCN^-, this series is identical to that described by Bormann *et al.* (10) for relative anion permeabilities through GABA-gated chloride channels. The neurochemical data presented here suggest that the relative potencies of competing organic anions such as picrate and niflumate are directly proportional to the permeability of the "competing" anion. Thus, both picrate and niflumate competed most efficiently (that is, were most potent in inhibiting anion-stimulated [^{35}S]TBPS binding) with the more permeant I^- and least efficiently with the less permeant F^-.

The deviation of SCN^- from the permeability sequence at GABA-gated chloride channels outlined by Bormann *et al.* (10) and Inomata *et al.* (12) might reflect a greater role for steric factors in the interaction of anions with an anion recognition site under the conditions used in neurochemical (disrupted cell membranes, static and symmetric ionic conditions across membranes) versus electrophysiological studies (whole cell or membrane patch, dynamic and asymmetric ionic compositions). Thus, under the former conditions, anion effects might be more indicative of their residence times at this putative anion binding site or their abilities to produce and maintain a conformation of the supramolecular complex more favorable to ligand binding. Under the latter conditions, anion effects may reflect their abilities to overcome the free energy barriers associated with channel traversal. In this regard, it should be noted that SCN^- has a substantially larger hydrated size than Cl^-, Br^-, or I^-, as well as a different charge geometry (linear versus spherical distribution for the halides). Although not deviating from the potency series outlined in this and electrophysiological studies, the behavior of F^- (biphasic effect, steeper Hill slope versus other anions; see Fig. 2) suggests that the relatively high electronegativity and low permeability and conductivity of this anion (10) may distinguish it from the other halides tested.

Significant differences were manifest in the manner by which picrate and niflumate inhibited anion-stimulated [^{35}S]TBPS binding (Table 2). Thus, both decreased the E_{max} of I^- to enhance [^{35}S]TBPS binding, whereas the E_{max} of Cl^- was unchanged or increased. Anion binding site heterogeneity offers a possible explanation for this data, inasmuch as differences in the affinities of halides for multiple anion binding sites might be revealed in the presence of the two organic anions. Alternatively, the effects of niflumate and picrate on anion-enhanced [^{35}S]TBPS binding may reflect the summation of their effects on the anion binding site and/or an allosteric site located proximally to it that is capable of differentially regulating the response of [^{35}S]TBPS binding. However, the inability of 11 other organic anions to produce similar effects indicates that the ability of picrate and niflumate to block the effects of small permeable anions on [^{35}S]TBPS binding is quite specific and not shared by other organic anions previously shown to affect the movement of anions through chloride channels that are not gated by GABA (23, 24).

Saturation analysis of the effects of picrate and niflumate on anion-stimulated [^{35}S]TBPS binding provided additional evidence consistent with an action of these organic anions at an anion binding site. In the absence of halides, [^{35}S]TBPS binding was very low (Table 1) (8). The addition of 200 mM Cl^- produced nearly a 6-fold increase in the B_{max} for [^{35}S]TBPS, which was in turn significantly decreased by both picrate (250 μM) and niflumate (100 μM; Fig. 3; Table 3). In addition, the 46% reduction in the K_d (increase in affinity) of [^{35}S]TBPS produced by Cl^- appeared to be reversed at least partially by these organic anions. *In toto*, these findings suggest a common site of action for permeant anions such as Cl^- and the larger organic anions picrate and niflumate.

Consistent with this hypothesis, picrate and niflumate also inhibited anion-promoted radioligand binding to benzodiazepine receptors without altering basal binding to these sites (Fig. 4). Thus, micromolar concentrations of picrate and niflumate reduced Cl^- (500 mM)-stimulated radioligand binding to benzodiazepine receptors by >50% (Fig. 4), exhibiting the same relative potency (niflumate > picrate) observed for blockade of anion effects on [^{35}S]TBPS binding. However, whereas the effect of both organic anions on [^{3}H]DMCM binding was apparent at all Cl^- concentrations, it was only apparent at higher anion concentrations in the case of [^{3}H]FNZ binding. This may be due primarily to the large difference in magnitude of the Cl^- effect on the binding of these two radioligands (the effect of Cl^- is approximately 10-fold greater on [^{3}H]DMCM binding than on [^{3}H]FNZ binding).

The effects of picrate and niflumate on GABA-ergic modulation of radioligand binding to benzodiazepine receptors were more complex. At concentrations (100–250 μM) that affected anion regulation of benzodiazepine receptors sites, the only significant effect was a small effect of picrate on the maximal stimulation of [^{3}H]FNZ binding by GABA. Much higher (2.5–5 mM) concentrations of picrate or niflumate produced mixed effects, changes in GABA potency that were not consistent between the two organic acids and radioligands tested (Table 4; Fig. 5, A and B). The relatively low potencies of both picrate and niflumate in affecting GABA-modulated versus chloride-

modulated radioligand binding to benzodiazepine receptors suggest that the site at which both anions act is distal to the GABA-binding portion of the supramolecular complex and more closely related to the anion binding site. The effects of high (millimolar) concentrations of the organic acids suggest that these agents may bind to additional sites and perturb other components of the complex, possibly by binding to other positively charged areas on this oligomeric protein. However, the large (20- to 25-fold) potency difference for these anions in exerting specific effects at or near the proposed anion binding site (inhibition of anion-enhanced TBPS and benzodiazepine receptor ligands) and other, perhaps less specific, effects (mixed effects on GABA-ergic modulation) still permits the use of appropriate low concentrations of niflumate and picrate as probes for the actions of anions on the GABA/benzodiazepine receptor supramolecular complex.

The recent report of the deduced amino acid sequence of a benzodiazepine and GABA receptor (25) suggests the presence of fixed cationic charges at locations thought to be near the opening of the chloride channel, a logical basis for the physical location of a putative anion binding site(s). The ability of low (micromolar) concentrations of organic anions such as picrate and niflumate to disrupt potently and selectively those radioligand binding interactions mediated by smaller permeant anions as well as to block the electrophysiological effects of such anions provides additional evidence that an anion binding site has an integral role in the function of the GABA/benzodiazepine receptor complex to regulate the movement of permeable anions across excitable membranes.

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