MOLECULAR PHARMACOLOGY, 20:470–476



# Halide- and γ-Aminobutyric Acid-Induced Enhancement of Diazepam Receptors in Rat Brain

## Reversal by Disulfonic Stilbene Blockers of Anion Channels

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Received October 17, 1980; Accepted June 8, 1981

SUMMARY

γ-Aminobutyric acid (GABA) and a few monovalent anions enhance benzodiazepine (BZ) binding to its receptor in rat brain by virtue of an increase in affinity. The effects of 4,4′-diisothiocyano-2,2′-disulfonic acid stilbene (DIDS) and 4-acetamido-4′-isothiocyano-2,2′-disulfonic acid stilbene (SITS), which are blockers of the anion channel in eyrthrocyte membranes, have been evaluated in this system. DIDS inhibits the increase in affinity of benzodiazepine receptors that is produced by maximal concentrations of GABA and iodide. DIDS also exerts a slight effect on control binding, due to a decrease in the number of sites without substantial change in apparent affinity. When membranes were pretreated with DIDS and then extensively washed, the effect of DIDS was reversible at 0° but irreversible at 37°. Dose-response studies of GABA and iodide using DIDS-pretreated membranes indicate that DIDS reduces the maximal response for GABA and decreases the sensitivity (increases the $ED_{50}$) for iodide. DIDS is more potent than SITS, which has weaker chloride channel-blocking properties. Four other amino-reactive reagents without anion channel-blocking properties failed to alter selectively BZ binding. These studies indicate that DIDS and related compounds may serve as useful probes of the interaction between the benzodiazepine receptor and the GABA receptor and further support the concept that these receptors are coupled to a mutually shared chloride channel.

### INTRODUCTION

Several lines of evidence demonstrate that the apparent affinity of BZ[3] for its receptor is modulated by GABA and by GABAergic agonists and antagonists (1–6). Tallman *et al.* (1) have postulated a direct interaction between GABA and BZ receptors at the postsynaptic membrane. A similar mechanism was proposed by Toffano *et al.* (7) and Guidotti *et al.* (8) on the basis of their finding of a protein (the so-called "GABA-modulin") which suppresses high-affinity GABA binding (7) and interacts with the BZ receptor (8). The affinity of the BZ receptor is also increased by a small group of monovalent anions (4, 9, 10). The three anions (chloride, iodide, and bromide) which have the greatest potency in enhancing BZ binding are the same three anions which are most active on GABA-dependent anion channels in various neurophysiological systems (10–13).

The facilitatory effects of anions and GABA on BZ receptor have features in common: (a) both are mediated by an increase in affinity; (b) both are temperature-dependent, being maximal at 0° and almost undetectable at 37°; and (c) both require the integrity of the membrane milieu of the BZ receptor, since BZ receptors solubilized by the detergent Lubrol lose responsiveness to GABA and iodide (14). However, the effect of the anions is not identical with that of GABA and varies in the following respects: (a) it is reduced by repeated washing of the membranes, a manipulation which enhances the effect of GABA; (b) it is profoundly decreased by Triton X-100 pretreatment, which produces the maximal activation by GABA; (c) it appears attributable to a reduction in dissociation rate for BZ, whereas an increase in the association rate is responsible for the effect of GABA (1); and (d) the stimulator effects of GABA and iodide are

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[3] The abbreviations used are: BZ, benzodiazepine; GABA, γ-aminobutyric acid; DIDS, 4.4′-diisothiocyano-2,2′-disulfonic acid stilbene; SITS, 4-acetamido-4′-isothiocyano-2,2′-disulfonic acid stilbene; DMS, dimethylsuberimidate; MAI, Methylacetimidate; *o*-MIU, *o*-methylisourea; PITC, phenylisothiocyanate; DME, *N,N′*-dimethylformamide.

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additive (1, 15, 16). These observations have been interpreted as a further indication that the interactions *in vitro* between the GABA receptor and the BZ receptor are due to their sharing of a common anion channel (9, 16). However, it has also been suggested that the effect of anions on BZ binding might be due to nonspecific chaotropic mechanism (10). Additional support for the physiological relevance of the effects of anions on the BZ receptor comes from the recent studies by Supavilai and Karobath (17). They have shown that a new group of anxiolytic agents enhances the affinity of the BZ receptor only in the presence of chloride, bromide, or iodide. The effect of these anxiolytic drugs and anions can be antagonized by picrotoxin, which is known to interact with GABA-dependent ionophores (13).

A specific monovalent anion transport system has been most extensively studied in erythrocytes (18, 19). The membrane constitutents involved in the transport process have been purified and identified, and the system has been reconstituted from isolated components (20, 21). A key factor in this successful development has been the use of the isothiocyano derivatives of disulfonic acid stilbenes, which have proven to be a unique class of highly site-specific membrane probes (22). DIDS has been reported to have specific effects in a wide range of experimental systems. Anion transport has been reported to be DIDS-sensitive in neurosecretory granules (23), epithelia (24), kidney slices (25), aplysia neurons (26), squid axons (27), Ehrlich ascites tumor cells (28), and even barnacle muscle (29). In the present study we examine the effects of DIDS on the enhancement of the affinity of the BZ receptor by GABA and anions.

## MATERIALS AND METHODS

Methods are essentially as described previously (9). All operations were performed at 4° unless otherwise indicated. Fresh brains (after removal of the cerebellum and medulla oblongata) from Sprague-Dawley rats were homogenized in 100 volumes of ice-cold Tris-maleate assay buffer (50 mM, pH 7.0 at 0°) with a Brinkman Polytron PT10 (15 sec at Setting 8). The homogenate was centrifuged at 20,000 × *g* for 20 min. For unwashed membrane preparations, the pellet was resuspended in the same buffer (100 volumes) and used in the binding assay. For washed membrane preparations, the pellet was rehomogenized in fresh buffer and recentrifuged five times before the final resuspension.

*Binding assay.* [$^3$H]Diazepam, 80 Ci/mmole, was purchased from New England Nuclear Corporation (Boston, Mass.). Triplicate incubation tubes (glass, 10 × 75 mm) packed in crushed ice received 0.1 ml of $^3$H-labeled ligand to give a final concentration 0.3 nM, 0.1–0.3 ml of various concentrations of drugs and/or salts, 0.3–0.1 ml of Tris-maleate buffer, and 0.5 ml of tissue suspension corresponding to 7.5 mg of the original tissue (wet weight). The total incubation volume was always 1 ml.

Tubes were incubated at 0° for 1 hr and then rapidly filtered under vacuum on Whatman GF/B filters with two 5-ml washes of ice-cold assay buffer. Filters were counted by liquid scintillation spectrometry in 10 ml of Hydrofluor (National Diagnostics Inc., Somerville, N. J.) in a Beckman $\beta$-counter at an efficiency of 38%. Alternatively, a Packard PRIAS spectrometer was used and filters were extracted in 5 ml of scintillation fluid in glass microvials.

Specific binding of diazepam was calculated as counts recorded minus counts recorded for "blanks" in the presence of 1 $\mu$M unlabeled benzodiazepine. DIDS, SITS, and other chemical probes were stored at −20° in darkness; they were weighed and dissolved in assay buffer immediately prior to use. Salt solutions were adjusted to pH 7.0 before use.

*Preincubation of membranes.* In experiments in which membranes were preincubated with probes (DIDS, SITS), homogenates were centrifuged and the pellets were resuspended in 40 volumes of buffer [37.5 mg of original tissue (wet weight) per milliliter] and incubated with the desired concentration of the probe. The pH, temperature, and length of these incubations are specified in the legends to the figures. Incubation was interrupted by centrifugation followed by a cycle of three resuspensions and recentrifugations (20,000 × *g* for 20 min) in buffers with or without bovine serum albumin (0.5%, w/v) as specified. These membranes were then used in the binding assay as described above. In each case, control membranes were subjected to an identical procedure with the exception of addition of the probe. DMS, MAI, and *o*-MIU were also examined in similar preincubation experiments. PITC was dissolved in a small volume of DMF and rapidly added to the aqueous membrane suspension with Vortex mixing.

*Calculations and data presentation.* Scatchard analysis was performed on data obtained from displacement isotherms using fixed concentrations of $^3$H-labeled ligand and varying amounts of unlabeled ligand. The results of these experiments were superimposible with experiments in which the $^3$H-labeled ligand was varied. Binding parameters (affinity and binding capacity) were obtained by computerized, weighted, nonlinear least-squares curve-fitting procedure (30).

## RESULTS

*Effect of antichaotropic anions (sulfate and fluoride) on the iodide stimulation of BZ binding.* Ammonium fluoride and sodium sulfate are known to be antichaotropic agents (31). These agents, even at a concentration of 200 mM, did not significantly affect control BZ binding (Fig. 1). Stimulation of BZ binding by iodide was not depressed in the presence of fluoride or sulfate. Instead, the effect of iodide was slightly increased by fluoride and significantly increased by sulfate (Fig. 1, *middle panels*). These effects of sulfate were specific for anion stimulation: neither fluoride nor sulfate affected the GABA stimulation of BZ binding. Guanidine HCl, a strong chaotropic agent, at concentrations between 1 and 200 mM had a deleterious affect on BZ binding in the absence of iodide (Fig. 1), with an $ID_{50}$ of 70 ± 10 mM. The inhibitory effect of guanidine HCl could be reversed in a dose-dependent fashion by sulfate (data not shown).

Figure 2 shows the dose-response curves for iodide stimulation of BZ binding with or without 200 mM ammonium sulfate. The sulfate effect was present for both washed and unwashed membranes. The iodide effect was greater in unwashed membrane preparations, especially

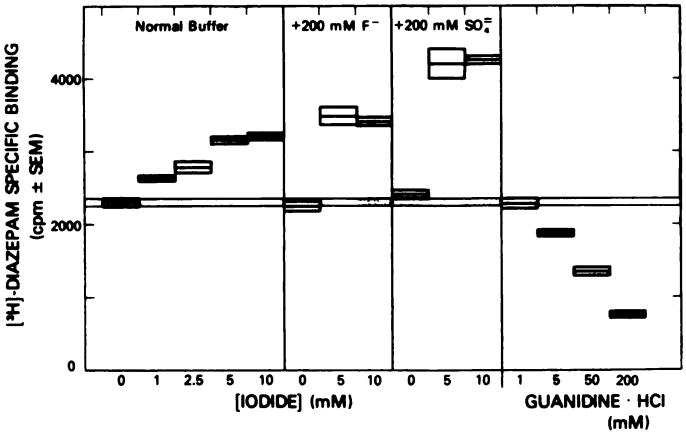


FIG. 1. *Dose-response curves for [³H]diazepam binding*

Left panels. Effect of 200 mM $F^-$ and $SO_4^=$ on dose-response curves for iodide stimulation of [³H]diazepam binding to washed membrane preparations. *Rectangles* indicate means ± 1 SEM; $n = 6$ for control group and $n = 4$ for other groups.

Right panel. Dose-response curve for the effect of guanidine HCl on [³H]diazepam binding.

in the presence of sulfate. The facilitatory effect of sulfate was most clearly seen at high concentrations of iodide. This finding suggests that the chaotropic effects of high concentrations of halide (which inhibit BZ binding) may be reversed by sulfate. The presence of 200 mM sodium sulfate appears to provide optimal conditions for studying the effects of halide on BZ binding.

*Effect of DIDS in the BZ-binding assay.* Figure 3A shows the effect of DIDS when present in the assay of [³H]diazepam binding to unwashed rat brain membranes. DIDS, at concentrations between 100 and 1000 μM, inhibited the iodide stimulation of BZ binding in both the presence (Fig. 3A, *middle panel*) and absence (Fig. 3A, *right panel*) of 50 μM GABA. A 100 μM concentration of DIDS reduced by 50% the stimulation of BZ binding produced by 25 mM KI alone or by 25 mM KI in the presence of 50 μM GABA. A 1000 μM concentration of

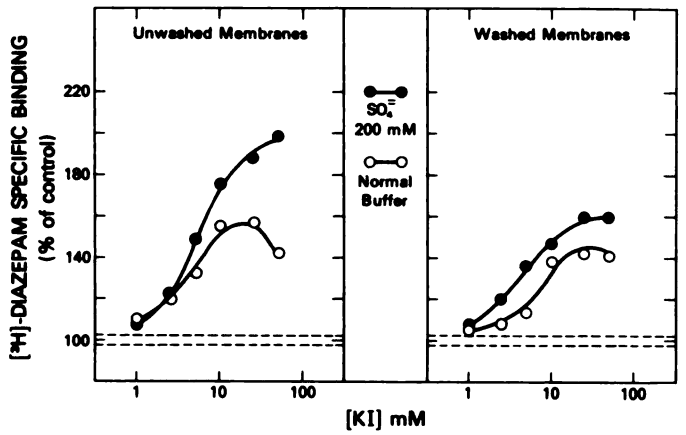


FIG. 2. *Effect of 200 mM sulfate on the dose-response curves for iodide stimulation of diazepam binding*

Sulfate enhances the effect of iodide in both washed and unwashed membrane preparations. Results shown are means of triplicates; - - -, mean ± 1 SEM for controls ($n = 9$).

DIDS blocked the effect of KI alone and inhibited the combined stimulation of KI and GABA by 80%. The same concentrations of DIDS did not significantly alter control binding (Fig. 3A, *left panel*) with the exception of a slight inhibition occurring at 1000 μM.

When washed membranes were used, the effect of DIDS on control binding was more prominent, with 30–40% inhibition of control BZ binding (Fig. 3B, *left panel*). Nevertheless, the ability of DIDS to block the effects of GABA or iodide is readily apparent (Fig. 3 B, *right panels*). Thus, both washed and unwashed membrane preparations show essentially the same dose-response curves to DIDS in the presence of iodide, GABA, and iodide plus GABA (Fig. 3A and B).

*Scatchard analysis of BZ binding effect of DIDS.* The effect of DIDS on GABA stimulation of BZ binding was studied in washed membranes (Fig. 4). A 50 μM concentration of GABA produced an increase in apparent affinity of the BZ receptor without a change in the number of sites ($R_o$), consistent with previous reports (1). DIDS (100 μM) significantly affected the control, producing a 30% reduction of the number of sites, but the affinity was not significantly affected. However, DIDS inhibited by approximately 80% the increase in affinity due to GABA. A pattern similar to that shown in Fig. 4 was also observed for iodide stimulation of BZ binding.

*Comparison of DIDS and SITS when present in the BZ binding assay.* Figure 5 shows the dose-response curves for DIDS and SITS in washed membranes. Both compounds were essentially equipotent in inhibiting BZ binding in the absence of GABA and iodide (*lower panel*). However, DIDS was more potent than SITS in reversing the GABA and iodide stimulation of BZ binding (*upper panel*). A similar pattern was also observed for iodide stimulation in the absence of GABA.

*Reversibility of the DIDS effect.* Membranes were washed once in buffer, and then were exposed to DIDS at 4° for 1 hr (corresponding to the standard binding assay conditions) or at 37° for 30 min. Control membrane preparations were preincubated in buffer in the absence of DIDS. Membranes were then extensively washed with buffer with or without 0.5 bovine serum albumin. (Albumin was included to serve as a low-affinity/high-capacity adsorbent to neutralize the probe.) [³H]Diazepam binding values in the two groups were compared.

When membranes were pretreated at 0° for 1 hr, GABA stimulated BZ binding to 60% above control, and 500 μM DIDS had no effect. GABA plus iodide stimulated BZ binding by 95%, and DIDS reduced this stimulation only slightly (to 80%).

In contrast, at 37° for 30 min, DIDS significantly reduced the effect of 50 μM GABA, from 70% to 20%. Likewise, the effect of GABA plus 25 mM iodide was reduced from 95% to 40%. Similar results were obtained whether or not albumin was included in the washing buffer. Hence, the effect of DIDS appears to be reversible at 0° but irreversible after 30 min at 37°.

*Comparison of DIDS and SITS in preincubation studies.* Membranes were washed with various concentrations of DIDS or SITS at 37° for 30 min. The membranes were then washed with albumin-containing buffers. The effects on BZ binding in the presence of GABA or GABA

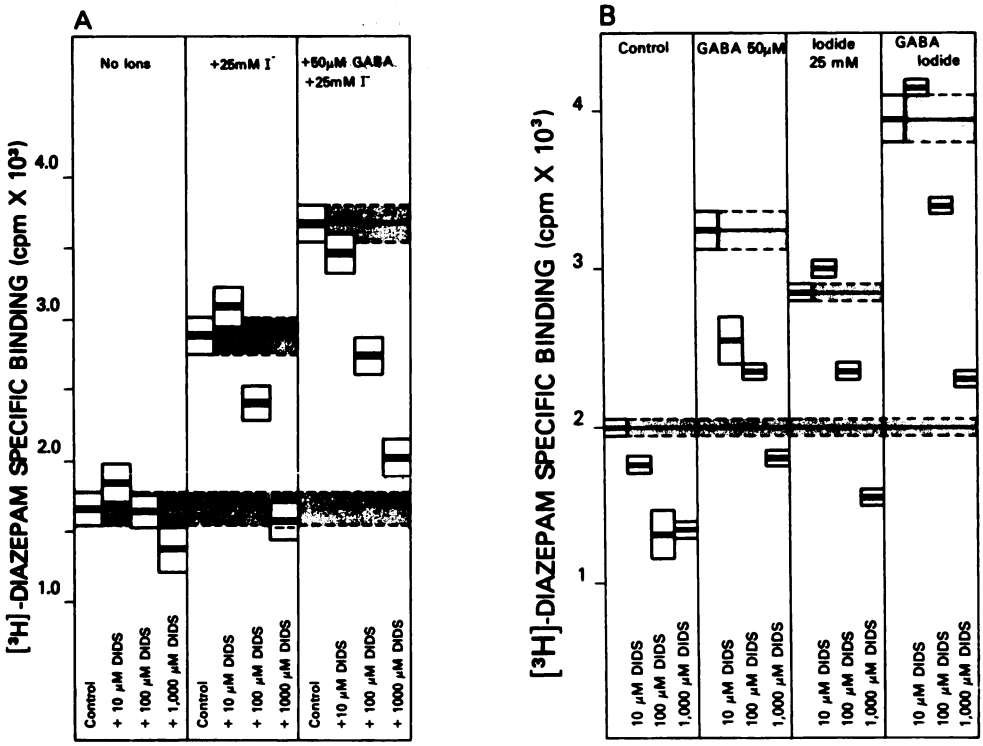


FIG. 3. *DIDS reverses the enhancement of diazepam binding by iodide and GABA*

A. Using unwashed membranes. *Left panel*, 1000 μM DIDS inhibits control BZ binding; *middle panel*, 25 mM iodide increases specific BZ binding by 75%, and the addition of progressively increasing concentrations of DIDS completely reverses the iodide effect; *right panel*, 25 mM iodide and 50 μM GABA result in further increases of BZ binding above control; again DIDS reverses this effect in a dose-dependent manner.

B. Using washed membranes.

and iodide were then examined (Fig. 6). Clearly, DIDS was more potent than SITS in preventing the stimulation of binding by either GABA or GABA combined with iodide.

*Effect of DIDS preincubation on the dose-response curve for GABA and iodide enhancement of BZ binding.* Figure 7 shows dose-response curves for the enhancement of [$^3$H]diazepam binding in response to GABA or iodide in membranes pretreated at 37°, with or without DIDS. The curve for GABA-stimulated binding shows a marked reduction in the upper plateau level in the DIDS-treated groups, whereas the $ED_{50}$ was not significantly altered (Fig. 7, *left panel*). The dose-response curve for iodide for the DIDS-treated membranes was displaced to the *right* and appears to be essentially parallel to the dose-response curve for untreated membranes (Fig. 7, *right panel*). A similar effect of DIDS on the dose-response curve for KI was seen in the presence of 5 μM GABA (data not shown). Similar results for both KI and GABA were also obtained when DIDS was present in the binding assay, using either washed or unwashed membranes.

*Specificity experiments.* Table 1 summarizes two experiments in which DMS, MAI, *o*-MIU, and PITC were used to pretreat the membranes. Although these compounds impaired control binding (suggesting that they were reactive under the conditions of the experiment), they did not inhibit the GABA-induced stimulation of BZ binding. *o*-MIU did not change the pattern of GABA stimulation, but it did produce a remarkable 2-fold increase in specific binding.

## DISCUSSION

*Chaotropic effects.* It is extremely unlikely that the stimulatory effect of iodide on BZ binding is due to a

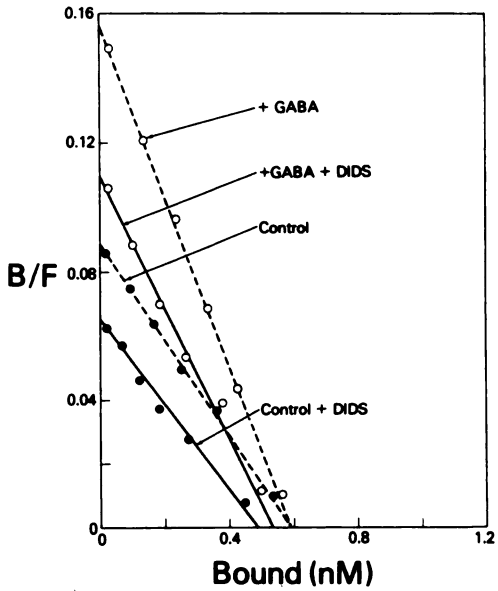


FIG. 4. *Effects of DIDS and GABA on the Scatchard plot of [$^3$H] diazepam binding to washed membrane preparations*

DIDS (100 μM) results in a reduction of $R_o$ with no significant effect on $K_d$. GABA (50 μM) significantly reduces $K_d$ with no effect on $R_o$. DIDS (100 μM) inhibits the GABA effect by increasing the $K_d$ value toward the control value.

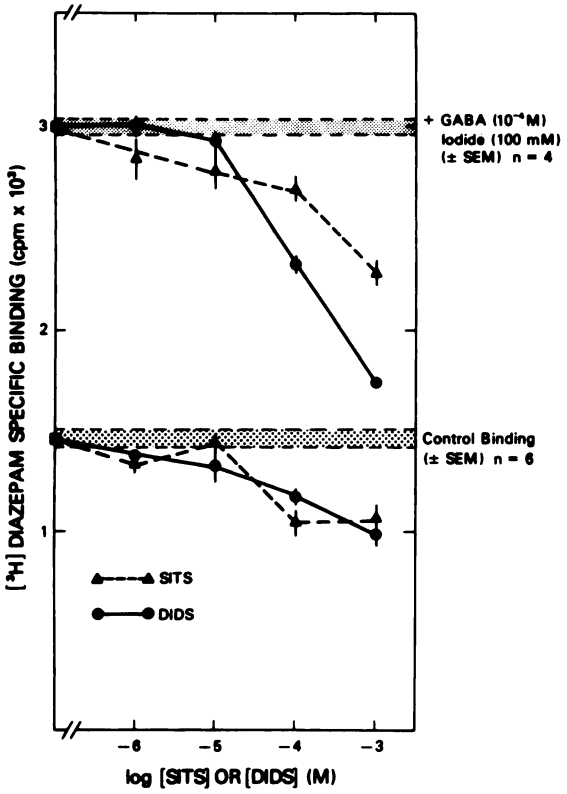


FIG. 5. *Effects of DIDS and SITS on [³H]diazepam binding to washed membranes*

Both DIDS and SITS show a dose-dependent inhibition of BZ binding in the absence of GABA or iodide (*lower panel*). DIDS and SITS are equipotent under control conditions; DIDS is more potent in the presence of GABA and iodide.

chaotropic effect: (a) antichaotropic ions ($F^-$, $SO_4^=$) actually enhance the iodide effect (Figs. 1 and 2); (b) strong chaotropic agents (guanidinium) inhibit BZ binding (Fig. 1); and (c) the "descending limb" of the dose-response curve for iodide (Fig. 2, *left*, and refs. 9) may be due to a chaotropic effect, since it is partially reversed by $SO_4^=$.

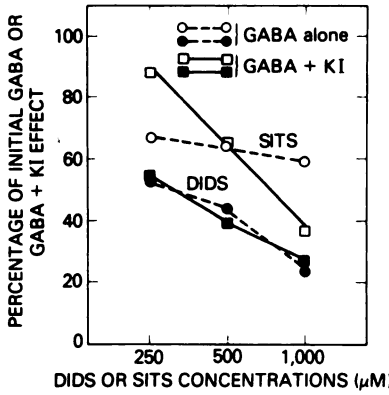


FIG. 6. *Effects of DIDS and SITS preincubation on GABA and iodide stimulation of [³H]diazepam binding*

DIDS and SITS were preincubated at 37° for 30 min with rat brain membranes, and then washed with albumin buffer as described. The percentage stimulation of GABA or of GABA plus iodide is shown as a function of DIDS or SITS concentration.

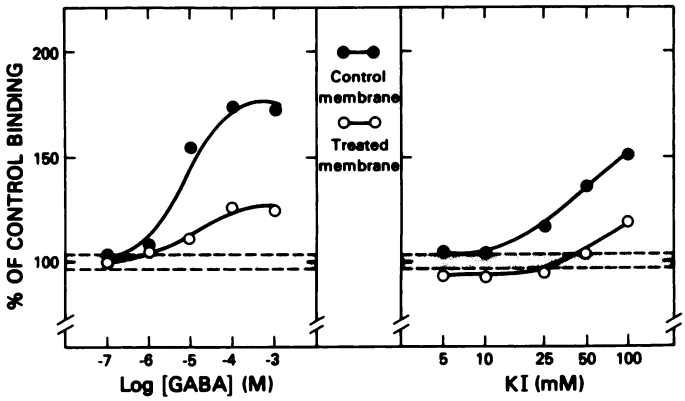


FIG. 7. *GABA and the effects of iodide on [³H]diazepam binding after DIDS preincubation*

Membranes were pretreated with DIDS as decribed under Materials and Methods. ●, Untreated membranes; ○, DIDS-pretreated membranes (100 μM).

*Effects of DIDS.* Clearly, DIDS has a dramatic effect, reversing the stimulation of BZ binding by GABA, iodide, or GABA and iodide (Fig. 3A, *left* and *right panels*). This effect is seen with the use of washed or unwashed membrane and is mediated by a signfificant change in $K_d$ with minimal effect on $R_o$ (Fig. 4). A similar effect is seen for SITS, at slightly higher concentration, when the compounds are added directly to the assay system or when they are preincubated with membranes. However, other compounds which might be expected to show similar

TABLE 1

*Effects of amino group modifiers on GABA-mediated stimulation of [³H]diazepam binding*

Experiment 1. Membranes were preincubated with DIDS or the indicated amino group reagent for 30 min at 37°, washed with albumin-containing buffer, and diluted for the [³H]diazepam-binding assay. Binding is expressed as percentage of the control binding to membranes preincubated with buffer alone. Data are means of quadruplicates (± 1 SEM) and are representative of two additional experiments.

Experiment 2. PITC was dissolved in DMF and added to the membrane suspension by vigorous stirring. An identical amount of DMF was added to the control membranes as well as to the membranes preincubated with DIDS. After 30 min at 37°, membranes were washed and assayed as described above. The nonspecific binding was significantly increased in the PITC-treated group and was 50% of the total binding.

| | [³H]Diazepam binding | [³H]Diazepam binding in presence of 50 μM GABA | % of net GABA effect |
|---|---|---|---|
| | *% of control* | *% of control* | |
| Experiment 1 | | | |
| Control | 100 ± 9 | 210 ± 100 | + 110 |
| DIDS (100 μM) | 96 ± 3 | 122 ± 1 | + 27 |
| DIDS (250 μM) | 85 ± 2 | 90 ± 7 | + 6 |
| DMS (1 mM) | 71 ± 5 | 133 ± 12 | + 87 |
| MAI (1 mM) | 104 ± 3 | 212 ± 5 | + 98 |
| *o*-MIU (1 mM) | 235 ± 8 | 469 ± 4 | + 100 |
| Experiment 2 | | | |
| Control (+ DMF) | 100 ± 7 | 156 ± 6 | + 56 |
| PITC (1 mM) | 51 ± 3 | 86 ± 29 | + 68 |
| DIDS (100 μM) | 87 ± 2 | 100 ± 6 | + 15 |

chemical reactivity with amino groups but lack the specific effect on the anion channel (DMS, MAI, *o*-MIU, PITC) do *not* inhibit the stimulation of BZ binding by GABA or GABA plus iodide. Thus, the effects of DIDS and SITS appear to be specific.

*Reversibility of DIDS preincubation experiments.* Under the conditions of the binding assay (0° for 1 hr) the DIDS effect is almost completely reversible. When DIDS is allowed to react with the membranes at 37°, a 30-min incubation period is sufficient to produce an effect which cannot be reversed by extensive washing with buffer or albumin solutions. The reversibility of the effect of DIDS in the ligand-binding system excludes the possibility that it is simply due to a nonspecific covalent reaction. The fact that the same action can be demonstrated when the probe is permanently linked to the membrane and the excess is removed seems to exclude other mechanisms such as direct interaction of DIDS with GABA. Preincubation of the membranes at 37° followed by extensive washing appears to provide the best conditions under which to demonstrate the DIDS inhibition of the iodide or GABA stimulation with minimal perturbation of the control binding.

*Comparison with erythrocyte system.* Erythrocyte membranes are equipped with a highly specialized device which allows them to transport anions at a rate several orders of magnitude higher than the rates of other cells. The transport mechanism has been identified as "band 3," a band of protein of 9,000 mol wt observed in sodium dodecyl sulfate-polyacrylamide gel electrophoresis of red blood cell membranes (32). Band 3 is also the binding site of DIDS in these membranes (33). It would be interesting to investigate whether a protein chemically related to band 3 might be present in certain central postsynaptic membranes. The stilbene-controlled anion transport in erythrocytes is an exchange flux, and, unlike the ligand-dependent anion channels in postsynaptic membranes, does not contribute to conductance. The large number of reports claiming an effect of DIDS in various systems (23–29) supports the concept that a general molecular recognition system might be involved in many anion-transporting membranes. An investigation of the neurophysiological properties of DIDS and congeners would also be desirable. A recent study reports that DIDS and SITS are potent blockers of an anion channel isolated from the electrical organ of *Torpedo californica* (34).

## CONCLUSION

Disulfonic stilbene probes, which are generally regarded as specific inhibitors of anion transport in erythrocytes, are able to antagonize GABA and iodide stimulation of BZ receptor binding. These compounds show several features which imply specificity in this system: (a) activity under both reversible or irreversible conditions; (b) expected relative potencies of DIDS and SITS (DIDS more potent than SITS); (c) consistent dose-response curves for effects, and specific effects on the appearance of the Scatchard plot; and (d) failure to mimic the effect with several nonspecific amino-reactive probes. These findings suggest the desirability of additional studies to examine similarities of ligand-dependent anion channels of inhibitory synapses of the central nervous system and the red blood cell anion-transport system.

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