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γ-Aminobutyric Acid Agonists and Antagonists Alter Chloride Flux Across Brain Membranes

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SUMMARY

γ-Aminobutyric acid (GABA), the major inhibitory neurotransmitter in the mammalian brain, increases membrane chloride conductance. Previously, we reported that GABA increases $^{36}Cl^-$ uptake by membrane vesicles (microsacs) prepared from mouse brain. Employing this technique, we found that the $GABA_A$ agonists, muscimol, isoguvacine, 4,5,6,7-tetrahydroisoxazolo(5,4-C)pyridine-3-ol, and 3-amino-1-propane sulfonate, all produced a concentration-dependent increase in $^{36}Cl^-$ influx, but baclofen, a $GABA_B$ agonist, failed to alter $^{36}Cl^-$ flux. Inhibition of GABA-dependent $^{36}Cl^-$ influx was produced by the convulsant drugs, bicuculline, picrotoxin, and pentylenetetrazole. Ion specificity was demonstrated by a failure of GABA agonists to stimulate influx of $^{45}Ca^{2+}$, $^{86}Rb^+$, $^{22}Na^+$, or $^{35}SO_4^{2}$. GABA-stimulated uptake of $^{36}Cl^-$ was largest in cortex and cerebellum and smaller in hippocampus and striatum. There was little difference in sensitivity to GABA among the areas. Analysis of subcellular fractions prepared from mouse brain demonstrated that the GABA-dependent $^{36}Cl^-$ influx was enriched in the synaptosomal fraction. The nonspecific (GABA-independent) uptake of $^{36}Cl^-$ was enriched in the myelin fraction. These experiments provide evidence for a functional coupling among GABA receptors and the chloride ionophore and suggest that the GABA-activated chloride channel is a site of action for several convulsant compounds.

Electrophysiological evidence suggests that the inhibitory effect of GABA on target neurons is primarily the result of an increase in chloride conductance (1–3). The increase in postsynaptic chloride permeability is generally assumed to be a rapid response to the interaction of GABA with its receptor, suggesting a close association of the GABA recognition site with the chloride ionophore. However, GABA produces both depolarization and hyperpolarization of nerve membranes, and it is not clear that both of these actions are due to changes in chloride conductance (4, 5).

Moreover, direct neurochemical evidence of chloride conductance changes following activation of GABA receptors is limited. For example, GABA has been reported to enhance $^{36}Cl^-$ conductance in rat hippocampal slices (6), embryonic chick neural cultures (7), and crayfish muscle (8). Although these findings offer proof of a GABA-stimulated chloride flux, there are several disadvantages to these techniques that limit their usefulness as tools in the study of receptor-mediated ion fluxes. For example, assay of embryonic tissue assumes that sufficient differentiation of the plasma membrane has occurred to provide the setting for GABAergic synapses and that these functions are representative of the adult neuronal system. In regard to the brain slice preparation, it is difficult to measure rapid ion fluxes because the outer portions of the slices are not viable, and large drug concentrations may be required to overcome the diffusional barriers that are intrinsic to the system. Membrane vesicles from rat brain have been used to demonstrate stimulatory effects of barbiturates on ^{36}Cl efflux (9, 10).

We recently demonstrated GABA-regulated chloride flux using a neural membrane preparation (11, 12). Our preparation (brain microsacs) contains sealed membrane vesicles with both pre- and postsynaptic elements (13). The GABA-stimulated uptake of $^{36}Cl^-$ by brain microsacs is quite rapid, with much of the influx occurring within 3 sec. Moreover, the system responds in a pharmacologically appropriate manner, lending further evidence that it will be useful for study of the GABA receptor-operated chloride channel. In this paper we characterize in detail the pharmacology of the GABA-stimulated $^{36}Cl^-$ flux of mouse brain microsacs.

Materials and Methods

Drugs. THIP was a gift of Dr. P. Krogsgaard-Larsen (Copenhagen, Denmark). Muscimol, GABA, APS, bicuculline, picrotoxin, and pentylenetetrazole were purchased from Sigma Chemical Co. (St. Louis,

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ABBREVIATIONS: GABA, γ-aminobutyric acid; P4S, piperidine-4-sulfonic acid; TBPS, *t*-butyl-bicylclophosphorothionate; EDTA, ethylenediaminetetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SITS, 4-acetamido-4′-isothiocyano-2-2′-disulfonic acid stilbene; APS, 3-amino-1-propanesulfonic acid; THIP, 4,5,6,7-tetrahydroisoxazolo(5,4-C)pyridine-3-ol.

MO). SITS was purchased from Calbiochem Behring Corp. (La Jolla, CA). $^{36}Cl^-$ in NaCl solution (0.2 μCi/ml) was purchased from ICN (Irvine, CA). Baclofen was a gift of Ciba-Geigy (Summit, NJ). Isoguvacine was obtained from Research Biochemicals, Inc. (Wayland, MA).

Bicuculline was dissolved in 0.1 N acetic acid. All other compounds were dissolved in distilled, deionized water. The pH of the solutions was adjusted to 7.0–7.5. All solutions were prepared immediately before use.

Membrane preparation. Male DBA/2N mice (National Cancer Institute Colonies), 60–120 days of age, were used in all experiments.

The procedure for preparation of the membrane vesicles (microsacs) was slightly modified from that described by Daly *et al.* (13). Mice were decapitated and the brains were rapidly removed and homogenized by hand (10–12 strokes) in 4.5 ml of ice-cold assay buffer (145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM D-glucose, 1 mM $CaCl_2$, and 10 mM HEPES adjusted to pH 7.5 with Tris base), using a glass-Teflon homogenizer (Thomas, size C). The homogenate was centrifuged at 2800 rpm (900 × g_{av}) for 15 min, using a Sorvall SA600 rotor. The supernatant was decanted and the pellet was resuspended in 8 ml of assay buffer and centrifuged at 2800 rpm (900 × gav) for 15 min. The final pellet was suspended in 7 ml of assay buffer, yielding a preparation containing 6–7 mg of protein/ml of suspension. Protein content was determined by the method of Lowry *et al.* (14). Exactly the same procedure was used for the preparation of a crude membrane suspension from mouse liver.

Subcellular fractions were prepared by a modification (15) of the procedure of Gurd *et al.* (16). Whole brain tissue from two mice was homogenized in 12.5 ml of ice-cold sucrose-HEPES buffer (3 mM HEPES, 0.32 M sucrose, 1 mM EDTA, pH 7.4) with a motor-driven Teflon-glass serrated homogenizer (Thomas, size C). The homogenate was centrifuged at 12,000 × g_{av} for 6 min. The resulting nuclear pellet (P_1) was washed once and retained on ice for influx studies. The pooled supernatants were centrifuged at 16,800 × g_{av} for 20 min to form the P_2 fraction. The resulting supernatant was centrifuged at 95,200 × g_{av} for 20 min to yield the microsomal fraction. The P_2 fraction was resuspended in 7 ml of sucrose-HEPES, and 5.5 ml were layered on a Ficoll density gradient (7.5 and 13%, w/v, in sucrose-HEPES) and centrifuged at 48,000 × g_{av} for 60 min in a Beckman SW27 rotor; the remaining 1.5 ml of P_2 was retained on ice until assayed. The synaptosomal layer was removed from the 7.5/13% Ficoll interface and the myelin fraction from the 0/7.5% Ficoll interface. The pellet was resuspended in 5 ml of sucrose-HEPES, overlayed on 1.3 M sucrose, 3 mM HEPES, pH 7.4, and centrifuged at 53,000 × g_{av} for 90 min in a Beckman SW27 rotor. The pellet was taken as the mitochondrial fraction (16). All of the fractions were washed once with buffer and resuspended in assay buffer to a concentration of 5–8 mg/ml of protein.

Procedure for $^{36}Cl^-$ uptake. Aliquots (200 μl) of membranes were incubated in a shaking water bath at 30° for a total of 10 min. Following this incubation, uptake was initiated by the addition and immediate vortexing of a 200-μl solution containing $^{36}Cl^-$ (0.2 μCi/ml of assay buffer). GABA agonists and SITS were added in the $^{36}Cl^-$ solution, except in the desensitization experiment (see Fig. 4), where muscimol was added 5 min before $^{36}Cl^-$ and with the $^{36}Cl^-$. Picrotoxin, bicuculline, and pentylenetetrazole were added 5 min before $^{36}Cl^-$. Three sec after the addition of $^{36}Cl^-$, influx was terminated by the addition of 4 ml of ice-cold assay buffer and rapid filtration under vacuum (10 in. Hg) onto a 2.4-cm Whatman GF-C glass microfiber filter, using a Hoefer manifold (Hoefer Scientific, San Francisco, CA). The filters were washed with an additional 8 ml of cold assay buffer with the filter towers removed. The amount of radioactivity on the filters was determined by liquid scintillation spectrometry. The amount of $^{36}Cl^-$ bound to the filters in the absence of membranes (no-tissue blanks) was subtracted from all values.

Calculation of $^{36}Cl^-$ uptake and statistical analysis. $^{36}Cl^-$ uptake was analyzed as being either GABA dependent or GABA independent. GABA-dependent uptake was defined as the amount of $^{36}Cl^-$ taken up while GABA was present in the medium (total uptake) minus the amount of chloride taken up when GABA was not present (GABA independent). Drug effects were analyzed statistically using an equal *n* multiple factorial repeated measures analysis of variance, unless otherwise noted in the text. Tukey A post-hoc analyses were run where appropriate and indicated.

Electron microscopy. Microsacs from whole mouse brain were prepared as described above and centrifuged at 14,000 × *g* for 5 min. The supernatant was decanted and the pellet was fixed in a 0.1 M cacodylate buffer containing 2.5% glutaraldehyde, 1% paraformaldehyde, and 4% sucrose at pH 7.4 for 1 hr at room temperature. The pellets were washed in 0.1 M cacodylate and centrifuged at 14,000 × *g* for 5 min and then treated with 2% osmium tetroxide for 45 min. The fixed tissue was washed with distilled water, embedded in Spurr's medium, and photographed through a Phillips EM 300 electron microscope.

Results

Morphologic examination of the microsac preparation. Morphological examination was carried out on pellets prepared from suspensions of microsacs. Electron microscope examination revealed a number of synaptic profiles (Fig. 1). Presynaptic profiles typically contained small dense vesicles, mitochondria, and few large clear vesicles. Frequently, the presynaptic profiles were attached to postsynaptic profiles with identifiable synaptic densities. Some free mitochondria, free nuclei, and broken myelin-like membrane structures were present. Several dozen sections were examined from the bottom, middle, and top of the pellets. No intact cells were observed in any of the fields examined.

Effect of GABA receptor agonists on $^{36}Cl^-$ uptake. The uptake of $^{36}Cl^-$ into whole mouse brain microsacs was stimulated by the $GABA_A$ agonists, isoguvacine, THIP, 3-APS, and muscimol (Fig. 2). Muscimol was about twice as potent as GABA at inducing $^{36}Cl^-$ influx, whereas THIP, isoguvacine, and APS proved less potent than GABA. These findings are in agreement with binding studies which suggest that muscimol has greater affinity, whereas THIP and APS have a lower affinity for the GABA recognition site compared with the natural ligand (17, 18). Isoguvacine and GABA were approximately equipotent in competing for the binding of [^{3}H]GABA, but isoguvacine was slightly less potent than GABA in stimulating chloride flux. Isoguvacine and muscimol were as efficacious as GABA and acted as full agonists in this system. In contrast, THIP and APS may be partial agonists (Fig. 2).

To test this hypothesis, microsacs were incubated with various concentrations of THIP and APS prior to muscimol stimulation of $^{36}Cl^-$ uptake. By definition, a partial agonist should attenuate the actions of a full agonist. APS clearly inhibited muscimol-induced $^{36}Cl^-$ uptake whereas THIP failed to attenuate muscimol-induced uptake (Fig. 3).

The $GABA_B$ receptor agonist, baclofen, failed to stimulate $^{36}Cl^-$ uptake at concentrations of 10, 100, or 1000 μM (data not shown). This is direct support for the suggestion that the $GABA_B$, or bicuculline-insensitive, receptor is not coupled with the chloride ionophore (19).

In the preceding studies, microsacs were exposed to GABA agonists for only 3 sec, because we observed in preliminary experiments that longer exposure decreased the effectiveness of the drugs. The possibility of receptor desensitization was investigated by exposing microsacs to various concentrations of muscimol for 5 min before measurement of muscimol-stimulated $^{36}Cl^-$ influx. Thus, microsacs were incubated with several

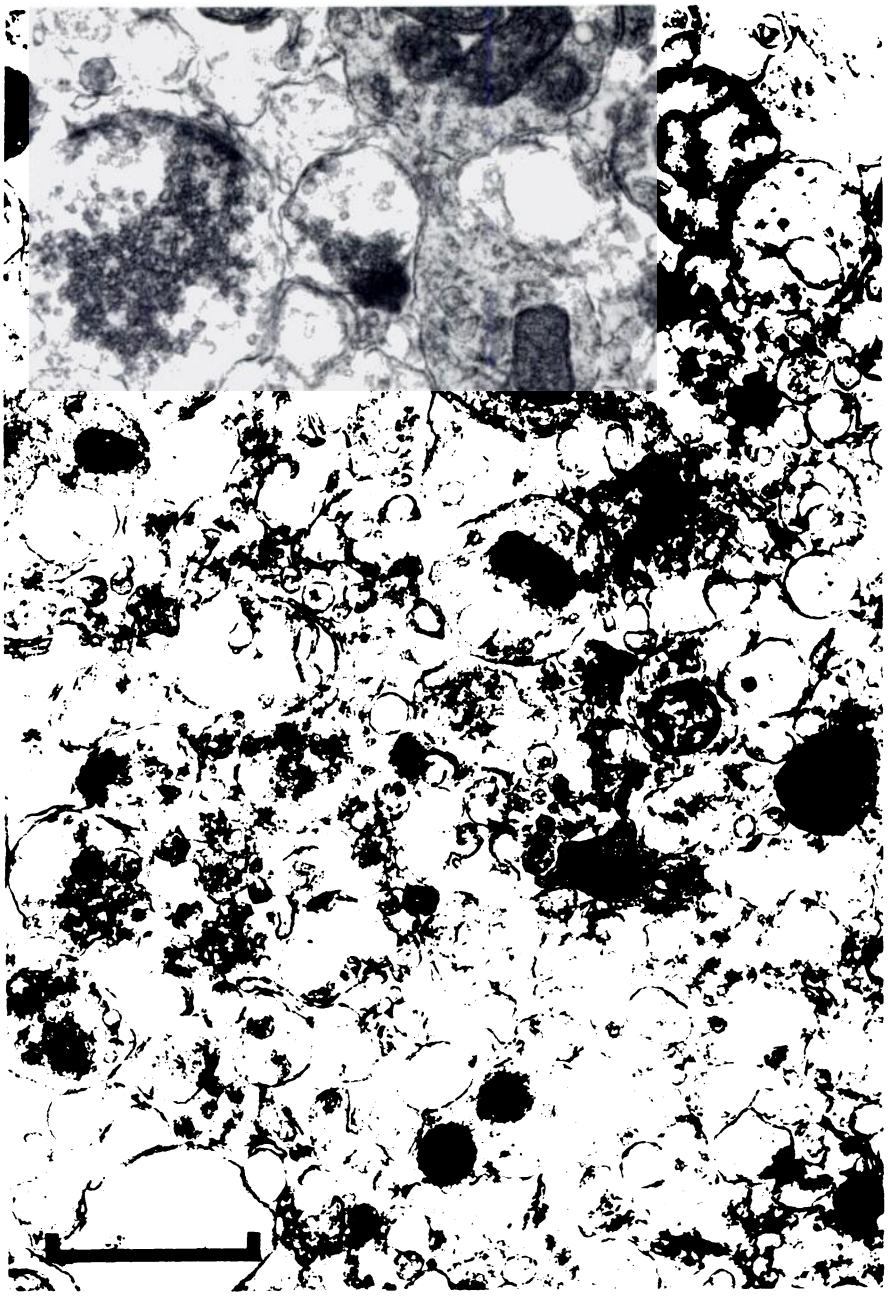
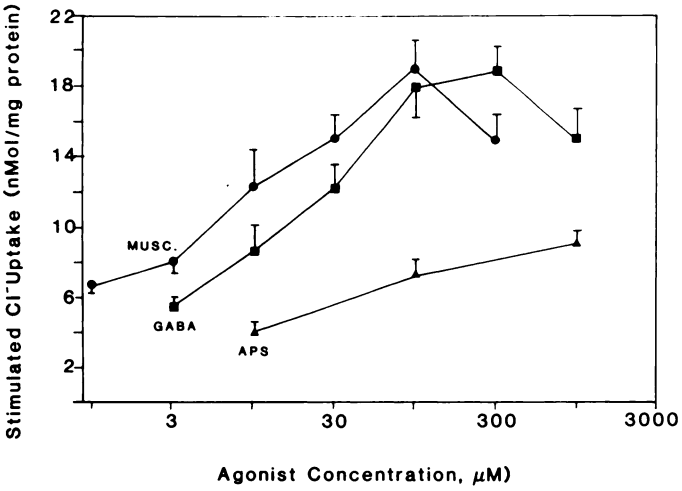


Fig. 1. Electron micrograph of microsacs prepared from whole mouse brain (see Materials and Methods). Pre- and postsynaptic elements and densities are clearly present. No intact cells were found. The *bar* represents 1 μm.

concentrations of muscimol, and uptake was initiated with the addition of a solution containing 30 μM muscimol and $^{36}Cl^-$. The longer exposure period markedly reduced the action of muscimol but did not alter basal uptake (Fig. 4). For example, preexposure to 2 μM muscimol reduced the action of 30 μM muscimol by about 40%. The desensitization appears to be competitive as preexposure to 2 μM muscimol reduced the effect by more than 80% when 4 μM muscimol was used to stimulate chloride uptake (data not shown). Desensitization following exposure to GABA has been observed in quench flow studies (11) and appears to be responsible for rapid inactivation of GABA-stimulated chloride flux (12). This requires that the assay be performed with a very short (3 sec or less) exposure to GABA. This is feasible without special equipment, and flux times of 1–5 sec are routinely used to study sodium and calcium fluxes across brain synaptosomes (20–22).

The effect of a 100 μM concentration of several amino acids (glutamate, aspartate, taurine, glycine, and diaminobutyric acid), and adenosine on $^{36}Cl^-$ uptake was also examined. None of these compounds increased chloride influx (Table 1), demonstrating that the action of GABA is specific for GABA agonists and is not shared by other amino acids. These data also illustrate the size of the signal and the amount of variability achieved with this assay. The maximal effect of GABA was approximately as large as the basal uptake (signal/noise = 1), and the SE was about 10% of the mean. The signal was not large, but the variability was sufficiently small to allow detection of a 20% change in basal flux. Measurement of small changes was facilitated by a repeated measures design where the same membrane preparation was tested under resting and stimulated conditions.

Effect of picrotoxin, bicuculline, and SITS on $^{36}Cl^-$



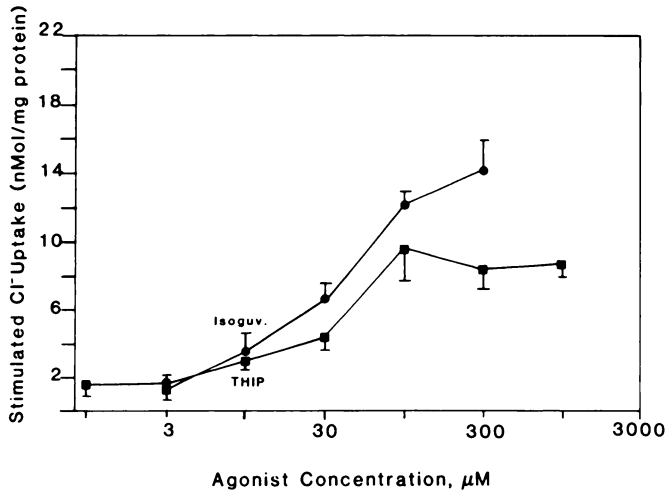


Fig. 2. Concentration-response curves for GABA (■, *upper panel*), muscimol (●, *upper panel*), APS (▲, upper panel), THIP (■, *lower panel*), and isoguvacine (●, *lower panel*) on $^{36}Cl^-$ uptake by mouse brain microsacs. Each *point* represents the mean (±SE) from eight different membrane preparations. The *abscissa* represents the agonist concentration (μM); the *ordinate* indicates the influx of $^{36}Cl^-$ (nmol/mg of protein/3 sec) produced by the agonists (uptake in the absence of agonist was subtracted from all values). Uptake time was 3 sec.

uptake. The GABA-stimulated influx of $^{36}Cl^-$ was blocked by prior exposure to low concentrations of either picrotoxin or bicuculline (Fig. 5). An attenuation of $^{36}Cl^-$ uptake of about 50% was obtained when a 10 μM concentration of the GABA receptor antagonist bicuculline was added to the microsacs. An even more substantial reduction of $^{36}Cl^-$ uptake was observed following the addition of 10 or 100 μM concentration of the chloride channel blocker, picrotoxin. The convulsant drug, pentylenetetrazole, also decreased GABA-dependent chloride flux, but was much less potent than bicuculline or picrotoxin (Fig. 6). The chloride transport inhibitor, SITS, produced a concentration-dependent decrease in the amount of $^{36}Cl^-$ uptake stimulated by GABA (Fig. 6). No decrease in $^{36}Cl^-$ uptake was produced by either picrotoxin, bicuculline, or pentylenetetrazole in the absence of GABA, suggesting that the microsac preparation does not contain sufficient endogenous GABA to activate the chloride channels. SITS was the only agent that inhibited flux in the absence of GABA, indicating that it is not selective for the GABA-activated chloride channel.

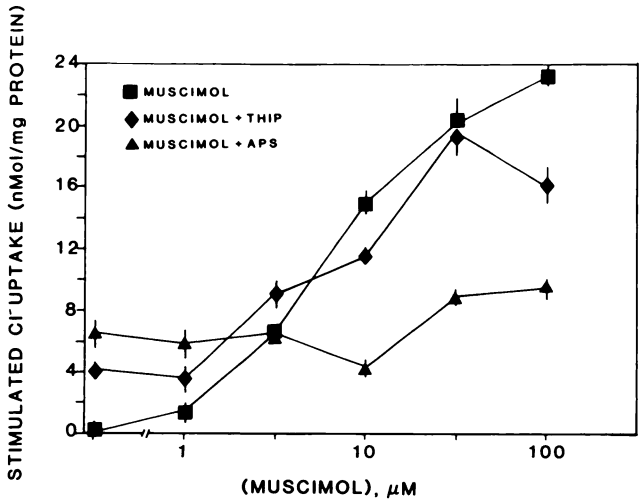


Fig. 3. Effects of THIP or 3-APS on muscimol-stimulated $^{36}Cl^-$ uptake. THIP (100 μM) or APS (300 μM) was added alone or with muscimol (concentration indicated on the *abscissa*). Drugs were present only during the 3-sec uptake period. The *ordinate* represents $^{36}Cl^-$ influx stimulated by the agonists (nmol/mg of protein/3 sec uptake time). ■, uptake in the absence of APS or THIP; ◆, microsacs incubated with THIP; ▲, incubation with APS. Values are means ± SE, $n = 4$. Analysis of variance revealed that APS significantly inhibited muscimol-stimulated uptake [$F(5,67) = 167$, $p < 0.0001$)]. THIP did not alter the action of muscimol ($p < 0.1$).

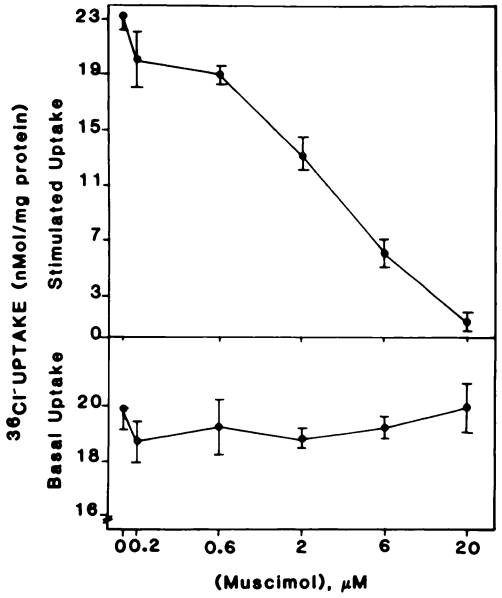


Fig. 4. Effects of preincubation with the GABA agonist, muscimol, on muscimol-stimulated Cl uptake. Muscimol was added 5 min before the initiation of uptake at the concentration indicated on the *abscissa*. The *ordinate* represents $^{36}Cl^-$ influx as nmol/mg of protein/3 sec uptake time. The *upper panel* represents the increment in uptake produced by addition of 30 μM muscimol with $^{36}Cl^-$. The *lower panel* is basal uptake (no muscimol added with $^{36}Cl^-$). Each *point* represents the mean ± SE, $n = 4$. Preincubation with muscimol significantly ($p < 0.01$) attenuated muscimol-stimulated uptake at all concentrations except 0.2 μM.

Effect of GABA receptor agonists on $^{36}Cl^-$ uptake in liver tissue. To test the possibility that nonspecific chloride trapping and non-neuronal chloride uptake might occur in our microsac preparation, we examined the effect of GABA and muscimol on $^{36}Cl^-$ uptake in a crude membrane suspension prepared from mouse liver. There was no evidence of stimulated

TABLE 1

Effect of amino acids and adenosine on $^{36}Cl^-$ influx by brain microsacs

Results are expressed as total $^{36}Cl^-$ uptake, nmol/mg of protein/3 sec, mean ± SE, $n = 4$. All compounds were tested at a concentration of 100 μM.

Compound	$^{36}Cl^-$ uptake
No addition	15.2 ± 1.0
GABA	30.1 ± 4.3
Glycine	15.3 ± 2.9
Aspartate	14.2 ± 8.4
Glutamate	14.1 ± 1.2
Taurine	14.8 ± 1.2
Diaminobutyric acid	14.1 ± 1.0
Adenosine	13.0 ± 0.6

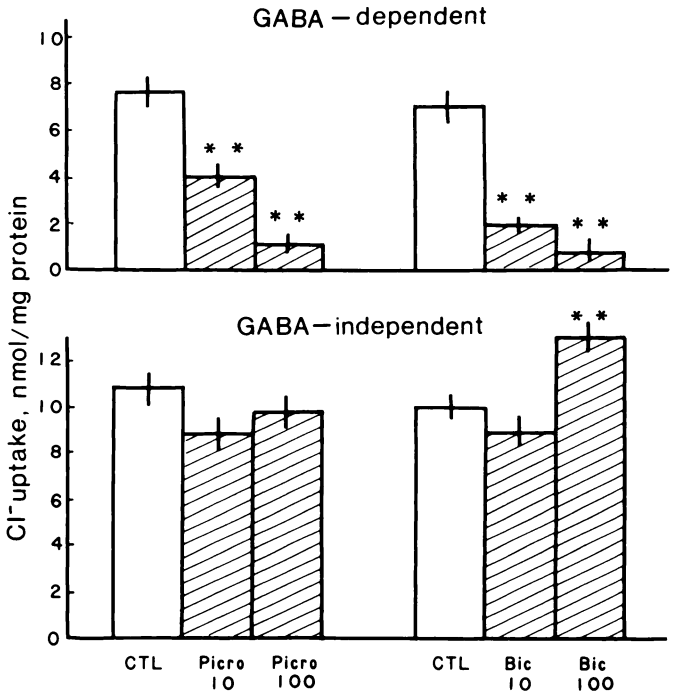


Fig. 5. Effect of the GABA antagonists, bicuculline and picrotoxin, on GABA-dependent (*upper panel*) and GABA-independent (*lower panel*) $^{36}Cl^-$ influx (nmol/mg of protein/3 sec). □, control (*CTL*): $^{36}Cl^-$ uptake in the absence of the antagonists. The concentration of GABA was 10 μM. Each *striped bar* represents mean ± SE, $n = 4$. **, significant difference from control, $p < 0.01$, paired *t* test. *Picro*, picrotoxin; *Bic*, bicuculline.

$^{36}Cl^-$ uptake in the liver membrane suspension following exposure to GABA (10 and 100 μM) or muscimol (3 and 30 μM) (Table 2). Other concentrations tested (GABA: 3, 30, μM, and muscimol: 1, 10 μM) also failed to stimulate uptake (data not shown).

Brain regional differences in GABA-stimulated $^{36}Cl^-$ influx. GABA-dependent chloride uptake was studied as a function of brain region. In general agreement with receptor binding studies using radioactive GABA agonists or convulsant drugs (23, 24), we found that GABA-dependent $^{36}Cl^-$ uptake varied in the following manner with brain region: cortex > cerebellum > hippocampus > striatum (Fig. 7). There was little difference among the brain regions in GABA-independent chloride flux (data not shown).

Subcellular analysis of GABA-stimulated $^{36}Cl^-$ influx. The highest density of GABA-binding sites is found in the synaptosomal fraction (25, 26). Similarly, subcellular fractionation shows that most of the GABA-dependent chloride uptake occurs in the synaptosomal fraction, whereas myelin had a large GABA-independent uptake of $^{36}Cl^-$ (Fig. 8). A comparison between the amount of GABA-specific $^{36}Cl^-$ uptake and nonspecific $^{36}Cl^-$ sequestering in the fractions indicated that the highest signal-to-noise ratio was found in the synaptosomes.

The effects of bicuculline and picrotoxinin on $^{36}Cl^-$ uptake stimulated by 10 μM muscimol were examined in brain synaptosomes. Both picrotoxinin and bicuculline produced a dose-dependent inhibition of muscimol-induced $^{36}Cl^-$ uptake (Table 3). Almost complete inhibition occurred at the highest antagonist concentration. Thus, in synaptosomes, as well as microsacs, the GABA-stimulated chloride influx is mediated by $GABA_A$ receptors.

Effect of GABA on the flux of other ions into microsacs. To investigate the possibility that the GABA-mediated uptake of $^{36}Cl^-$ is the result of either a nonspecific ion flux or a breakdown in membrane integrity, the uptake of $^{45}Ca^{2+}$, $^{86}Rb^+$, $^{22}Na^+$ and $^{35}SO_4^{2-}$ was measured in the presence of GABA receptor agonists. GABA (30 or 300 μM) failed to produce any significant uptake of $^{45}Ca^{2+}$, $^{86}Rb^+$, or $^{35}SO_4^{2-}$. This is in clear contrast to the effects of GABA on $^{36}Cl^-$ uptake (Fig. 9). There was, however, some apparent uptake of $^{22}Na^+$ with the highest concentration of GABA. This uptake of $^{22}Na^+$ was not observed with similar concentrations of muscimol (Fig. 10), suggesting that the uptake of $^{22}Na^+$ was not due to a GABA receptor-operated ionophore, as was the case with $^{36}Cl^-$. It is likely that the observed uptake of $^{22}Na^+$ was due to co-transport of GABA and sodium.

Influence of temperature on $^{36}Cl^-$ uptake. The amount of $^{36}Cl^-$ uptake was measured in microsacs after 10 min incubation at several temperatures in the presence or absence of 10 μM GABA. Incubation temperatures ranging from 20 to 40° appeared to have little effect on GABA-stimulated $^{36}Cl^-$ flux (Fig. 11). Increasing the temperature to 45° did, however, result in an increase in GABA-independent $^{36}Cl^-$ uptake.

Discussion

Actions of GABA have been divided into "bicuculline-sensitive" and "bicuculline-insensitive" (27), and these appear to correspond to interaction with $GABA_A$ and $GABA_B$ receptors, respectively (19). We conclude that the GABA-stimulated chloride flux measured in our experiments was due to activation of $GABA_A$ receptors because it was antagonized by bicuculline and produced by $GABA_A$ agonists, but not by the $GABA_B$ agonist, baclofen. Bicuculline-sensitive, GABA-dependent, fluxes of $^{36}Cl^-$ have also been demonstrated in monolayer culture of chick embryo neural tissue (7) and in hippocampal slices (6). These studies are basically in agreement with our results, although there are some differences. The concentration of GABA reported for half-maximal $^{36}Cl^-$ efflux by Wong *et al.* (6) was 400 μM compared with an EC_{50} of about 20 μM in our system, and the EC_{50} of 6 μM reported by Thampy and Barnes (7).

We found that the GABA receptor agonists, muscimol, isoguvacine, THIP, and APS all stimulated $^{36}Cl^-$ influx. The relative potencies of these agonists were generally consistent with their potencies in competing with either [^{3}H]GABA for the high affinity GABA receptor (18) or in competing with [^{3}H] bicuculline methochloride for the low affinity receptor (28). However, the concentrations of agonists required for enhancement of chloride flux were about 1000-fold higher than the

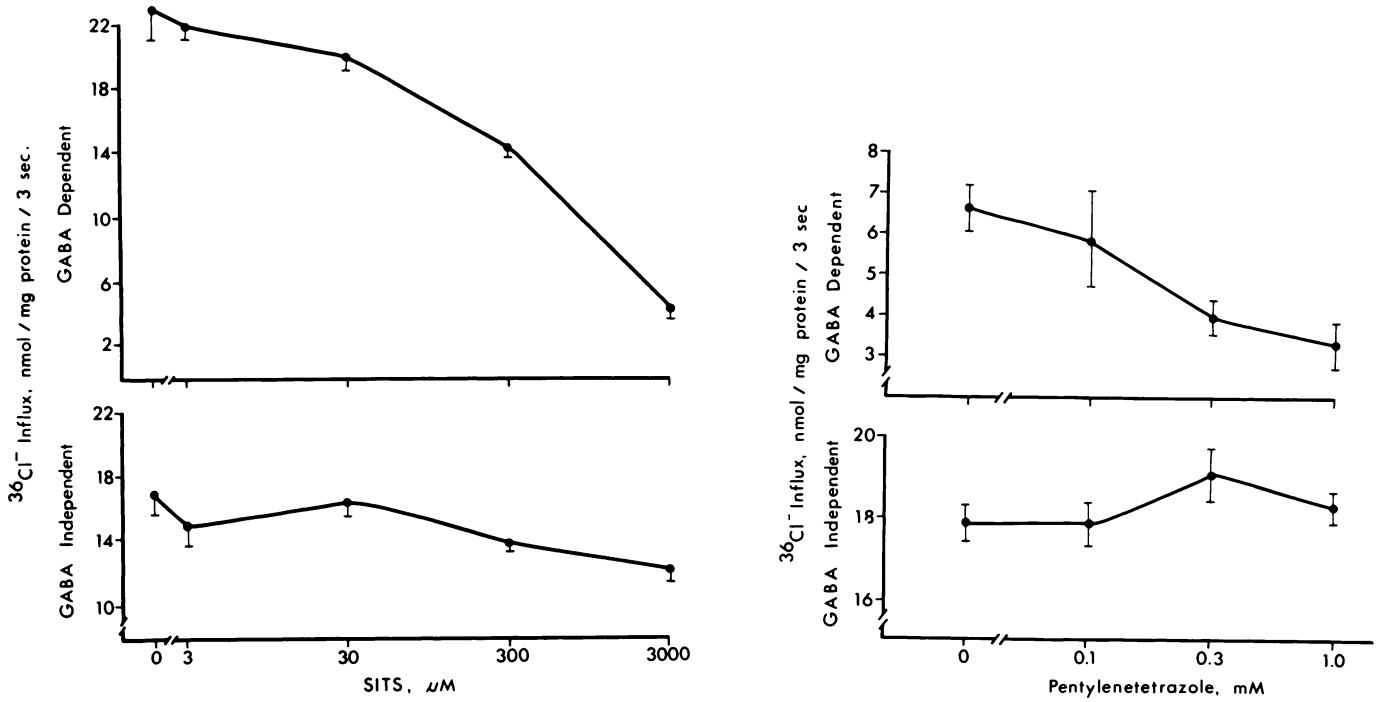


Fig. 6. Effects of pentylenetetrazole and SITS on GABA-dependent (*upper panels*) and GABA-independent (*lower panels*) $^{36}Cl^-$ uptake (nmol/mg of protein/3 sec). The concentration of GABA was 10 μM for pentylenetetrazole and 100 μM for SITS. Each *point* represents mean ± SE, $n = 4$. Pentylenetetrazole produced a dose-dependent decrease in $^{36}Cl^-$ uptake indicated by the presence of a dose × presence or absence of GABA interaction. $F(3,28) = 7.4$, $p < 0.005$. SITS also inhibited uptake: $F(4,15) = 67.4$, $p < 0.001$. GABA produced a significant stimulation of uptake with all drug concentrations.

TABLE 2

Effect of GABA agonists on $^{36}Cl^-$ uptake by liver membranes

Results are expressed as total $^{36}Cl^-$ uptake, nmol/mg of protein/3 sec, mean ± SE, $n = 4$.

Compound	$^{36}Cl^-$ uptake
No addition	2.00 ± 1.2
GABA (10 μM)	−2.05 ± 2.73
GABA (100 μM)	−4.85 ± 1.7
Muscimol (3 μM)	−0.92 ± 0.99
Muscimol (30 μM)	0.81 ± 1.14

concentrations required for inhibition of the high affinity GABA binding and 100-fold higher than the concentrations needed to compete for the low affinity site (18, 28, 29). As discussed previously (12), it is likely that this discrepancy arises because binding studies detect only high affinity desensitized states of the receptor, whereas flux studies measure the affinity of the active state of the receptor. Desensitization of the GABA-dependent chloride flux was demonstrated directly in the present study by preincubation with muscimol. The concentrations of muscimol required to produce desensitization were lower than those required to stimulate $^{36}Cl^-$ influx, again suggesting that desensitization involves a higher affinity state of the receptor than is required for channel activation. GABA response desensitization has also been demonstrated in electrophysiological studies of hippocampus (30).

The maximal stimulation of chloride flux was similar for GABA, muscimol, and isoguvacine. This indicates that muscimol and isoguvacine act as full agonists in this assay. However, THIP and APS did not stimulate chloride flux to the same extent as the other agonists. In the present study, APS clearly acted as a partial agonist by antagonizing muscimol stimulation of $^{36}Cl^-$ uptake, whereas THIP failed to attenuate or to augment

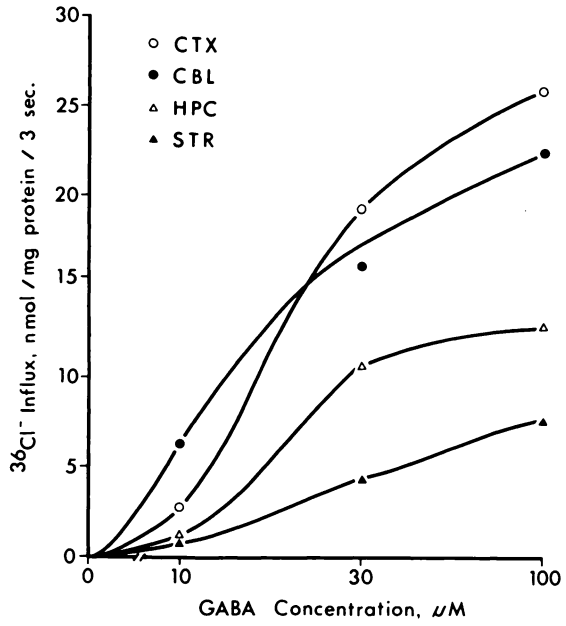


Fig. 7. Brain regional analysis of GABA-stimulated uptake of $^{36}Cl^-$ by microsacs prepared from mouse cortex (*CTX*, ○), cerebellum (*CBL*, ●), hippocampus (*HPC*, △), and striatum (*STR*, ▲). The *ordinate* represents $^{36}Cl^-$ uptake (nmol/mg of protein/3 sec). The *abscissa* represents the concentration of GABA. Each *point* represents the mean ± SE, $n = 4$.

the effect of muscimol (the latter would be expected if THIP were a full agonist). [^{3}H]THIP has 90% fewer high affinity binding sites compared with [^{3}H]GABA and [^{3}H]P4S (31). Thus, the low efficacy of THIP in the present study could be a reflection of its ability to interact with only a fraction of the sites available to GABA or muscimol. It is interesting to note

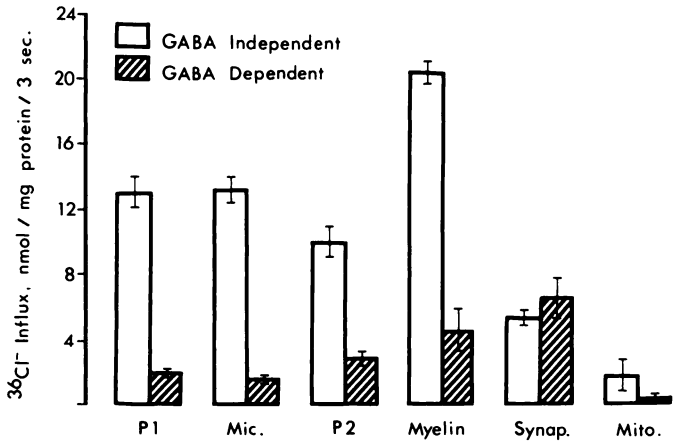


Fig. 8. Subcellular analysis of GABA-dependent (▨) and GABA-independent (□) uptake of $^{36}Cl^-$. Uptake (nmol/mg of protein/3 sec), indicated on the *ordinate* for subcellular fractions [P_1, microsomes (*Mic.*), P_2, myelin, synaptosomes (*Synap.*), and mitochondria (*Mit.*)], were prepared from mouse brain. The concentration of GABA was 10 μM. Values are means ± SE, $n = 8$.

TABLE 3

Effects of muscimol, bicuculline, and picrotoxin on uptake of $^{36}Cl^-$ by isolated brain synaptosomes

Results are expressed as muscimol-dependent uptake of $^{36}Cl^-$, nmol/mg of protein/3 sec, mean ± SE, $n = 4$.

Compound	$^{36}Cl^-$ uptake
Muscimol (10 μM)	5.00 ± 1.0
Muscimol + bicuculline (10 μM)	4.70 ± 0.8
Muscimol + bicuculline (100 μM)	−2.00 ± 0.6*
Muscimol + picrotoxinin (10 μM)	3.90 ± 0.4
Muscimol + picrotoxinin (100 μM)	0.50 ± 0.7*

* Significantly different from muscimol alone, $p < 0.05$.

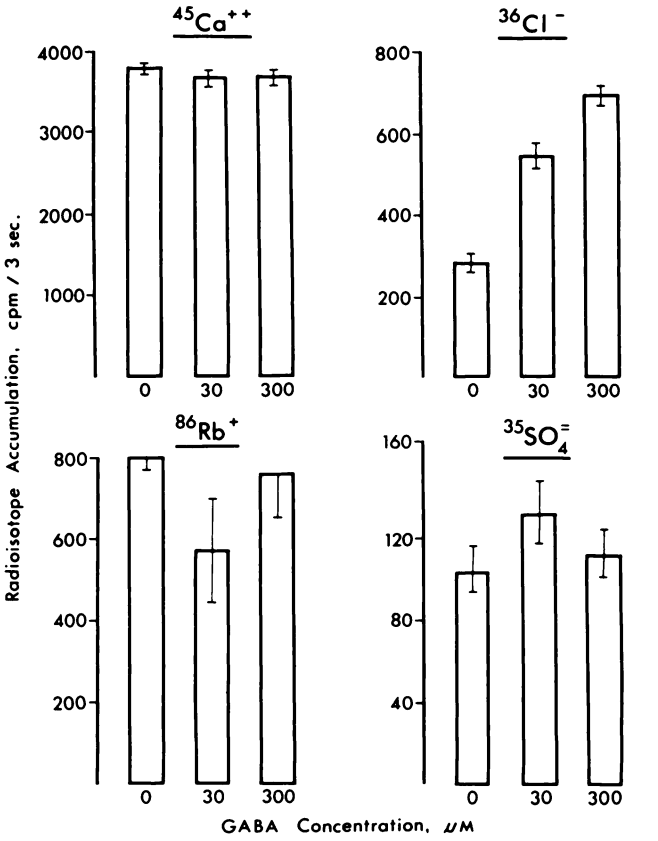


Fig. 9. Effects of GABA on the uptake of $^{45}Ca^{2+}$, $^{36}Cl^-$, $^{86}Rb^+$, or $^{35}SO_4^=$ by brain. The *abscissa* represents radioisotope accumulation as cpm/sample for an uptake time of 3 sec. The total cpm added to each sample was 1×10^6 (^{45}Ca), 0.8×10^6 (^{36}Cl), 1×10^6 (^{86}Rb), and 0.7×10^6 ($^{35}SO_4$). The *abscissa* gives the GABA concentration in μM. Each value is the mean ± SE, $n = 4$. Only $^{36}Cl^-$ accumulation was altered by GABA, $F(2,12) = 8.4$, $p < 0.001$.

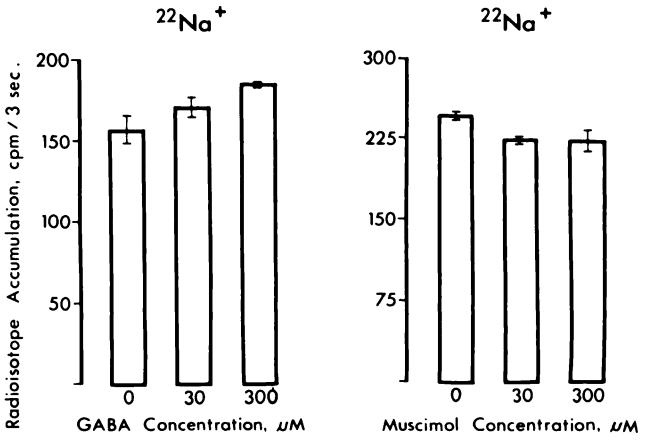


Fig. 10. Effects of GABA (*left*) and muscimol (*right*) on the accumulation of $^{22}Na^+$ by brain microsacs. The *ordinate* represents $^{22}Na^+$ accumulation (cpm/sample) in 3 sec. The total cpm added to each sample was 380,100 ($^{22}Na^+$). The *abscissa* gives agonist concentration in μM. Each *bar* represents mean ± SE, $n = 4$. The only significant difference observed was a stimulation $^{22}Na^+$ uptake by 300 μM GABA, $t(6) = 3.34$, $p < 0.02$.

that THIP and APS also have low efficacy in enhancing the binding of [^{3}H]diazepam to brain membranes, but muscimol acts as a full agonist in this assay (18, 32). These observations suggest that there are similar agonist requirements for optimal coupling of the GABA receptor with either the chloride channel or the benzodiazepine receptor.

GABA produces both hyperpolarizing and depolarizing responses in brain. The hyperpolarization is attributed to chloride influx, and the depolarization may be due to chloride efflux or cation influx (27). Our experiments measured $^{36}Cl^-$ influx, but this indicates only that the chloride channel is open and not the direction of *net* chloride flux. In several systems, THIP is more potent than GABA for hyperpolarization but less potent for depolarization (5, 11). In our studies, THIP was less potent than GABA, suggesting a depolarizing action. Alger and Nicoll (4, 5) proposed that depolarization by GABA (and THIP) is due to cation influx mediated by dendritic receptors. However, GABA failed to stimulate $^{45}Ca^{2+}$ or $^{86}Rb^+$ flux, and muscimol failed to stimulate $^{22}Na^+$ flux, suggesting that any functional coupling of the GABA receptor with channels for these cations is not detectable in our system.

Measurement of GABA-stimulated $^{36}Cl^-$ uptake in subcellular fractions prepared from brain demonstrated significant activity in all fractions except mitochondria. However, the synaptosomal fraction displayed both the largest uptake (per mg of protein) and the highest ratio of GABA-dependent to GABA-independent uptake. The pharmacology of the synaptosomal chloride uptake was similar to that of microsacs and consistent with coupling of synaptosomal chloride channels with $GABA_A$ receptors. All subcellular fractions except mitochondria also displayed uptake of $^{36}Cl^-$ in the absence of GABA. This GABA-independent uptake is probably non-neuronal as

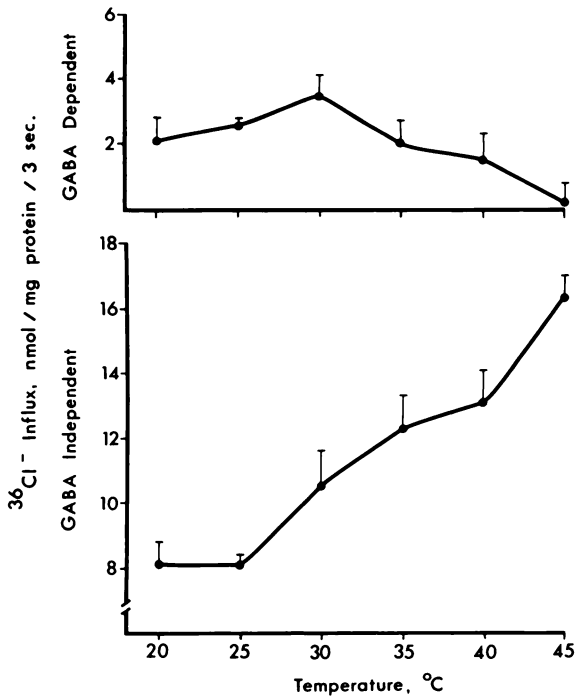


Fig. 11. Effect of incubation temperature on GABA-dependent (*upper panel*) and GABA-independent (*lower panel*) $^{36}Cl^-$ uptake. The *ordinate* represents $^{36}Cl^-$ influx (nmol/mg of protein/3 sec). The concentration of GABA was 10 μM. The *abscissa* represents the temperature at which microsacs were incubated for 10 min prior to initiation of uptake. An effect of temperature, $F(5,18) = 5.53$, $p < 0.004$, as well as a temperature × GABA presence or absence, $F(5,18) = 11.18$, $p < 0.002$ was observed. Each *point* represents mean ± SE, $n = 4$. GABA produced a significant stimulation of uptake at all temperatures except 45°.

it was enriched in the myelin fraction and low in the synaptosomal fraction.

The temperature dependence of the chloride flux was also studied. GABA-independent uptake of $^{36}Cl^-$ increased markedly with temperature, suggesting active transport of chloride, but GABA-dependent uptake changed very little from 20 to 40°. The latter result is somewhat surprising because, even though one expects the GABA-activated flux to be a passive process, the binding of GABA to the receptor and the activation of the channel should vary with temperature (33). Our techniques did not allow us to examine the thermodynamics of each of the steps involved in activation of the channels, and it is possible that the temperature dependence of the steps balance so that there is little temperature dependence for the overall process.

The inhibition of GABA-stimulated $^{36}Cl^-$ uptake by pentylenetetrazole is consistent with electrophysiologic studies employing this convulsant. Pentylenetetrazole blocks GABA-mediated responses in a noncompetitive manner (34) and inhibits the enhancement of GABA binding produced by pentobarbital without affecting basal GABA binding (35). It appears that pentylenetetrazole may be acting at the picrotoxin recognition site, since it competitively inhibits [^{35}S]TBPS binding (36). The concentrations of pentylenetetrazole required to inhibit chloride flux in the present study were similar to the brain levels (330 μM) of the drug required to produce convulsions (37). Thus, three different convulsants, pentylenetetrazole, picrotoxin, and bicuculline, all inhibited the GABA-mediated chloride flux.

The chloride transport inhibitor, SITS, attenuated both GABA-independent and -dependent $^{36}Cl^-$ uptake. SITS appears to be a rather nonselective inhibitor of chloride fluxes as it inhibits GABA-induced depolarization of dorsal root ganglia (38) and antagonizes stimulation of [^{3}H]diazepam binding by iodide or GABA (39), in addition to its well known inhibition of Cl^-/HCO_3^- exchange (40). SITS binds covalently to membrane proteins following long incubation times (41), but inhibition of Cl^- flux by SITS also occurs by a rapid, noncovalent interaction. For example, a 42% inhibition of Cl^- flux 1 min after a 10 μM concentration of SITS has been reported for the LRM55 glioma cell line (42). The inhibition of GABA-stimulated $^{36}Cl^-$ flux detected in the present study within 3 sec following SITS probably represents noncovalent interaction.

In summary, assay of $^{36}Cl^-$ flux across brain membranes provides a functional measurement of actions of GABA agonists and antagonists. We used this assay to determine the potency of these drugs, to distinguish between full and partial agonists, to study the concentration dependence of agonist-antagonist interactions, and to provide information about desensitization of the GABA-stimulated chloride flux. This assay system can be combined with quench flow techniques to provide detailed information about the kinetics of channel activation and receptor desensitization (11).[1]

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