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Differences in the negative allosteric modulation of γ-aminobutyric acid receptors elicited by 4′-chlorodiazepam and by a β-carboline-3-carboxylate ester: A study with natural and reconstituted receptors

(benzodiazepine/chloride channels/cDNA transfection)

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ABSTRACT **Cl^- currents elicited by γ-aminobutyric acid (GABA) application were recorded with the whole-cell tight-seal technique from voltage-clamped cortical neurons of neonatal rats in primary culture. The peripheral benzodiazepine recognition site ligand 4′-chlorodiazepam [Ro 5-4864; 7-chloro-1,3-dihydro-1-methyl-5-(4-chlorophenyl)-2*H*-[1,4]-benzodiazepin-2-one] inhibited the GABA-generated currents in a dose-dependent manner. Also, a β-carboline (DMCM; 6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate methyl ester), acting as a negative allosteric modulator of $GABA_A$ receptors, reduced the intensity of GABA-generated currents with similar efficacy but greater potency. Flumazenil (Ro 15-1788; 8-fluro-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo-[1,5-*a*][1,4]-benzodiazepine-3-carboxylate ethyl ester) antagonized DMCM inhibition but not that elicited by 4′-chlorodiazepam. The isoquinoline carboxamide PK 11195, an antagonist of 4′-chlorodiazepam effects in other systems, failed to antagonize the action of 4′-chlorodiazepam. The transient expression of various molecular forms of $GABA_A$ receptors in the human embryonic kidney cell line 293 allowed a study of the minimal structural requirements for the inhibition of GABA-induced Cl^- currents by bicuculline, picrotoxin, 4′-chlorodiazepam, and DMCM. GABA-elicited Cl^- currents in cells coexpressing α_1 and β_1 subunits of $GABA_A$ receptors were inhibited by bicuculline and picrotoxin, but not by DMCM or 4′-chlorodiazepam. Conversely, the GABA currents in cells coexpressing $\alpha_1\beta_1$ and γ_2 subunits were inhibited by bicuculline, picrotoxin, 4′-chlorodiazepam, and DMCM. Since the Cl^- currents generated by GABA in some molecular forms of $GABA_A$ receptors are inhibited by bicuculline and picrotoxin only, 4′-chlorodiazepam cannot be acting isosterically with picrotoxin.**

The $GABA_A$ receptors include in their structure an allosteric modulatory site that regulates the probability that γ-aminobutyric acid (GABA), acting on its specific recognition site, evokes Cl^- currents. There are three types of ligands for such a modulatory site: the anxiolytic benzodiazepines, which increase the probability of GABA action (positive allosteric modulators); the various anxiogenic derivatives of β-carboline-3-carboxylate, which decrease the probability of GABA action (negative allosteric modulators); flumazenil, which is practically devoid of intrinsic activity but antagonizes the action of positive and negative allosteric modulators and is a pure antagonist of the modulatory site.

The diazepam derivative 4′-chlorodiazepam has a pharmacological profile different from that of its parent compound. Although these two drugs share a sedative action (1) possibly related to a decrease of neurotransmitter release resulting from a reduction of presynaptic calcium entry (2), their action on $GABA_A$ receptors differs. The anticonvulsant diazepam increases the probability that GABA evokes Cl^- currents, while the proconvulsant 4′-chlorodiazepam decreases such a probability.

In spite of the chemical similarity between 4′-chlorodiazepam and diazepam, the former binds with an affinity lower than that of the latter to the "classical" benzodiazepine recognition site (3). Moreover, 4′-chlorodiazepam displaces picrotoxin better than diazepam does (4), even though its affinity for this site is lower than that for the benzodiazepine recognition site found in peripheral tissues (5).

Despite the original belief that 4′-chlorodiazepam binds selectively to nonneuronal binding sites (6), a negative modulatory action on GABA-activated Cl^- channels was recorded from neurons of mammalian central nervous system (7) and of ciliary ganglion (8). Recent biochemical and anatomical evidence (9, 10) suggests that a novel regulatory site linked to $GABA_A$ receptors mediates the effect of 4′-chlorodiazepam.

The availability of cDNA clones for various structural subunits of $GABA_A$ receptor (11, 12) and the possibility of transiently expressing these cDNAs (13) in human embryonic kidney cells (293 cell line) allows the investigation of $GABA_A$ receptors assembled from different structural subunits. While GABA, bicuculline, and picrotoxin act on receptors formed from α_1 and β_1 subunits (13, 14), the coexpression of these subunits with the γ_2 subunit is required to express the positive or negative allosteric modulation of GABA-evoked Cl^- currents by either benzodiazepine or β-carboline derivatives (12).

In various molecular forms of recombinantly expressed $GABA_A$ receptors, we compare the negative modulation of GABA-elicited Cl^- currents by 4′-chlorodiazepam to that of the β-carboline derivative 6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate methyl ester (DMCM) and to the inhibition by picrotoxin. Furthermore, we analyze whether flumazenil, the antagonist of the allosteric modulatory site (15), inhibits the action of these two compounds in cortical neurons and in the 293 cell line, transiently expressing different combinations of the $GABA_A$ receptor subunits.

MATERIALS AND METHODS

Primary Culture of Cortical Neurons. Primary cultures of neonatal rat cortical neurons were prepared as described (16). Briefly, the cells were dispersed by using 0.25 mg of trypsin

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Abbreviations: GABA, γ-aminobutyric acid; DMCM, 6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate methyl ester.

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(Sigma) per ml and plated at a density of 0.8–1 × 10^6 on 35-mm Nunc dishes previously coated with poly(L-lysine) (Sigma) at 10 μg per ml. The cultures were kept in the incubating medium consisting of basal Eagle's Medium, 10% (vol/vol) fetal bovine serum (GIBCO), 25 mM KCl, 2 mM glutamine (Sigma), and 100 μg of gentamicin (GIBCO) per ml for 1–3 weeks. After 24 hr *in vitro*, the medium was replaced, and 1 μM cytosine arabinofuranoside was added to inhibit replication of nonneuronal cells.

Culture from Kidney Embryonic Cell Line and cDNA Transfection. Transformed human embryonic kidney cells 293 (ATCC CRL 1573) were grown in minimum essential medium (MEM, GIBCO) supplemented with 10% fetal bovine serum containing 100 units of penicillin (GIBCO) and 100 units of streptomycin (GIBCO) per ml in a 6% CO_2/94% air incubator. Exponentially growing cells were trypsinized and seeded at 2 × 10^5 per 35-mm dish in 2 ml of growth medium. The transfection was performed by using the calcium phosphate precipitation technique (17). The cloned cDNAs of human $GABA_A$ receptor subunits α_1, β_1, and γ_2 (12, 18), inserted singly or together into the eukaryotic expression vector pCIS2 (13), were used to perform the transfection. The cells were incubated in the presence (3 μg per 35-mm dish) of one or two supercoiled plasmids for 12–16 hr at 37°C under 3% CO_2/97% air. The medium was removed, and the cells were rinsed twice with growth medium, refed, and incubated in the same medium for 24 hr at 37°C under 6% CO_2/94% air before electrophysiological studies.

Electrophysiology. The single-electrode voltage-clamp technique in the whole-cell configuration (19) was used to study primary cultures of cortical neurons (1–3 weeks) or transfected cells (24 hr after transfection) on the stage of an inverted microscope (Zeiss IM-35) at room temperature. The recording pipette contained either 145 mM potassium gluconate/1.1 mM EGTA/2 mM $MgCl_2$/10 mM Hepes·NaOH at pH 7.2 or 145 mM CsCl/1 mM $MgCl_2$/11 mM EGTA/10 mM Hepes·CsOH at pH 7.2. Cells were bathed in 145 mM NaCl/5 mM KCl/2 mM $CaCl_2$/5 mM Hepes·NaOH, pH 7.4. Osmolarity was adjusted to 325 milliosmolar with sucrose. The liquid-junction potential between the recording pipette and ground was corrected by using a saline bridge connected to ground that contained the two solutions. GABA (0.5 M in H_2O, pH 4 with HCl) was applied by iontophoresis by using 30-ms pulses of positive current. GABA iontophoretic currents in the 25- to 50-nA range were used to generate outward currents in neurons in such a way as to obtain a peak amplitude of 150–200 pA. 4'-Chlorodiazepam and flumazenil were from Hoffman–La Roche, and DMCM was from Ferrosan (Copenhagen). The benzodiazepine and β-carboline derivatives were dissolved in bath solution containing dimethyl sulfoxide at a maximal final concentration of 0.01%. These drugs were applied by pressure (2–4 psi; 1 psi = 6.89 kPa) in the proximity of the cell body with micropipettes of 5 to 10 μm diameter.

Applying dimethyl sulfoxide (0.01% in medium) failed to modify the GABA responses. To avoid uncontrolled drug leakage, this pipette was kept outside the bath before the pressure injection and brought in proximity to the recorded cell just before drug application. 4'-Chlorodiazepam and DMCM were applied for 3 s between two GABA pulses delivered every 10 s. Current traces were amplified by a patch-clamp amplifier (EPC-7, List Electronics, Darmstadt, F.R.G.) filtered at 1500 Hz (eight-pole low-pass bessel, Frequency Devices, Haverhill, MA) and were recorded on a chart recorder (Gould 2600S) for off-line analysis.

Often the GABA response was greater in cells adjacent to those being recorded from. This observation might relate to electrical coupling among 293 cells (13). This feature made it easier to find cells expressing the $GABA_A$ receptor subunits in the culture dish. Recordings in this preparation could be performed until day 3 after transfection.

RESULTS

GABA-Evoked Cl^- Currents in Cortical Neurons: Effects of 4'-Chlorodiazepam and DMCM. Iontophoretic applications

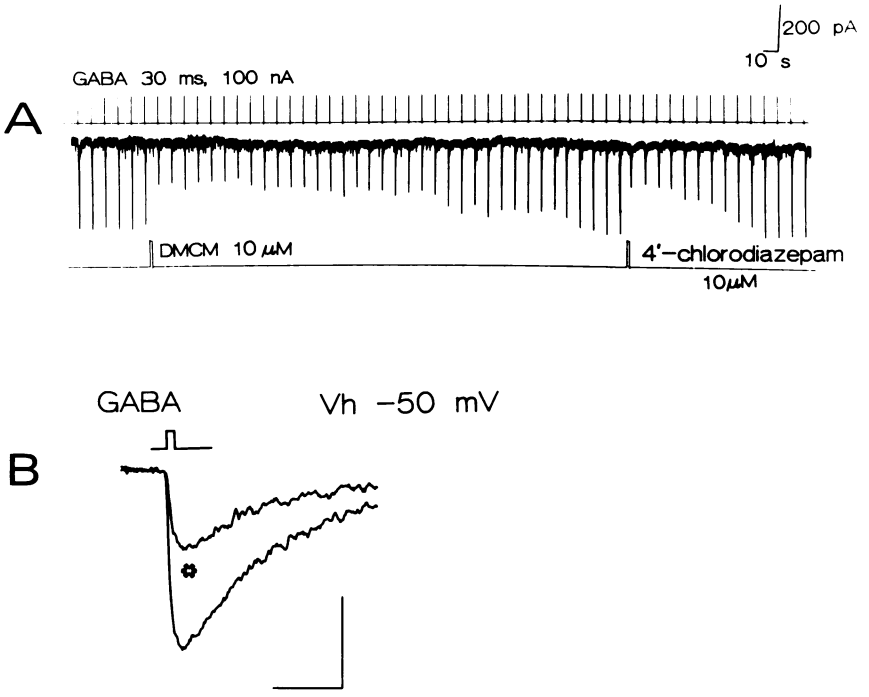

FIG. 1. Antagonism of GABA-evoked Cl^- currents by 4'-chlorodiazepam and by DMCM in cortical neurons. (*A*) The two drugs were pressure-ejected for 3 s between GABA pulses, and both inhibited inward Cl^- currents (seen as downward deflections) in a reversible manner. Symmetrical chloride was used, and the holding potential was −60 mV. (*B*) Inhibition of GABA-activated currents by 4'-chlorodiazepam (*) seen on a faster time scale. (Calibration = 100 ms, 200 pA.)

of GABA evoked Cl^- currents in voltage-clamped cultured cortical neurons when the membrane potential was clamped at −60 mV (close to the measured resting potential; ref. 16). When the pipette solution contained Cl^- as a permeable anion, the equilibrium potential for Cl^- was close to 0 mV, and the current measured was inward. Pressure applications of DMCM produced a fast decrease of these GABA-evoked inward Cl^- currents. This effect was dose dependent and slowly reversible after washing (Fig. 1*A*). Also, applications of 4′-chlorodiazepam produced a fast, dose-dependent decrease of the Cl^- currents readily reversible upon washing (Fig. 1*B*).

The dose dependence of the antagonism of GABA-elicited Cl^- currents by 4′-chlorodiazepam and DMCM is shown in Fig. 2. The fitting of these dose–response curves with a sigmoid relationship yields an IC_{50} of 250 nM for DMCM and an IC_{50} of 3 μM for 4′-chlorodiazepam. Conversely, the maximal decrease of the Cl^- currents observed with both compounds was similar even though the lack of saturation of the 4′-chlorodiazepam dose–response does not allow efficacy evaluation.

To investigate the voltage-dependence of the antagonism produced by 4′-chlorodiazepam, we performed voltage-clamp experiments with a recording pipette containing gluconate as the major anion in which we measured the intensity of GABA-elicited Cl^- currents at different holding potentials in the presence and absence of 4′-chlorodiazepam (see Fig. 3). The degree of antagonism of the GABA response appeared to be similar at each holding potential—i.e., the reduction of the GABA-activated Cl^- current produced by 4′-chlorodiazepam was not voltage dependent. Similar results were observed when the reduction of Cl^- current was produced by DMCM (data not shown).

Antagonism of the Negative Modulation of GABA-Elicited Cl^- Currents. When DMCM was pressure-applied from a pipette that also contained flumazenil, an isosteric antagonist of the benzodiazepine- and β-carboline-mediated allosteric modulation of GABA-elicited Cl^- currents, its effect on GABA currents was completely antagonized (Fig. 4). Flumazenil (1 μM) has no intrinsic effect on GABA responses and slightly potentiates GABA responses at higher micromolar concentrations (20). On the contrary, when flumazenil is applied simultaneously with 4′-chlorodiazepam, the antagonism of GABA-activated Cl^- current was unaffected. A comparison of the results obtained when flumazenil was combined with 4′-chlorodiazepam or DMCM, shows that the benzodiazepine antagonist selectively inhibited the response elicited by DMCM but failed to modify the inhibition of 4′-chlorodiazepam. Following a report that the peripheral benzodiazepine receptor ligand PK 11195 antagonized the action of 4′-chlorodiazepam (21), we tried to counteract the action of 4′-chlorodiazepam with PK 11195. We failed to antagonize the negative modulation of GABA-induced Cl^- currents by 4′-chlorodiazepam with PK 11195 (Fig. 5). At present no specific antagonist is known for the 4′-chlorodiazepam-mediated inhibition of GABA-evoked Cl^- currents.

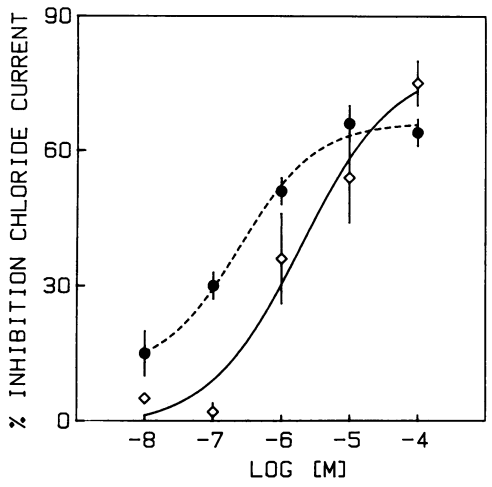

FIG. 2. Dose-dependent antagonism of GABA-activated Cl^- currents recorded in cortical neuron in culture by DMCM (●) and 4′-chlorodiazepam (◇). DMCM shows lower efficacy when compared with 4′-chlorodiazepam (EC_{50} DMCM = 250 nM; EC_{50} 4′-chlorodiazepam = 3 μM).

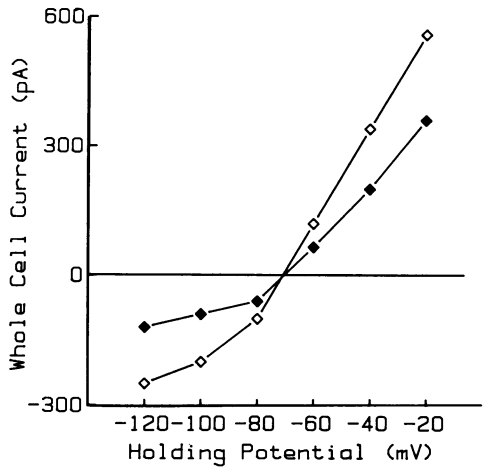

FIG. 3. Current-to-voltage relationship for whole-cell GABA-activated Cl^- current in cortical neurons in the presence (◆) and absence (◇) of 4′-chlorodiazepam (10 μM). In this experiment potassium gluconate was the major salt in the recording pipette, producing a GABA current reversal potential of −76 mV.

4′-Chlorodiazepam and DMCM on GABA-Evoked Cl^- Currents in Transfected 293 Cell Line. Iontophoretic applications of GABA evoked inward flow of Cl^- in cultured 293 embryonic kidney cells transiently transfected with $GABA_A$ receptor subunits α_1 and β_1 and voltage-clamped at −40 mV in a symmetrical Cl^- solution. The Cl^- currents elicited by GABA in these cells were antagonized by 20 μM bicuculline methiodide (three cells tested per each $GABA_A$ receptor subunit combination) and were not observed in nontransfected cells. We tested 4′-chlorodiazepam and DMCM on the

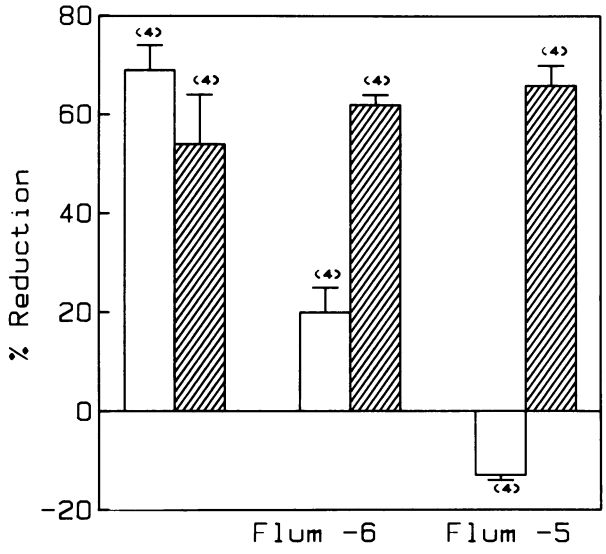

FIG. 4. Reduction of GABA-activated Cl^- currents after application of 10 μM DMCM (□) or 10 μM 4′-chlorodiazepam (▨) in the presence and absence (set of bars on the left) of flumazenil (Flum). The concentration of flumazenil is expressed as the log of the molar concentration.

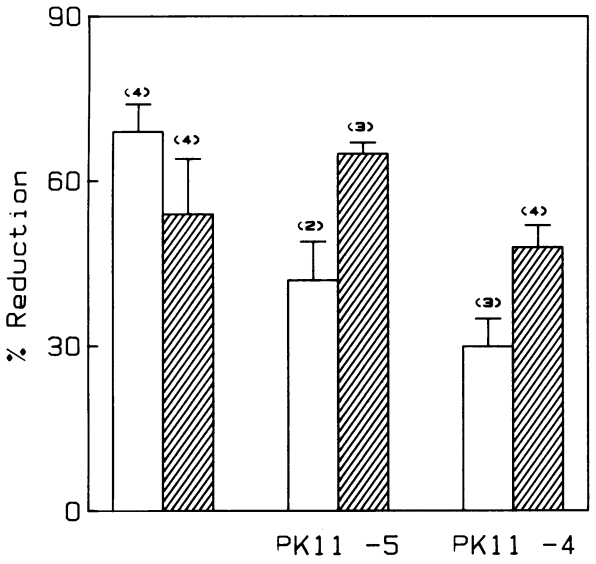

FIG. 5. Reduction of GABA-activated Cl^- currents after application of 10 μM DMCM (□) or 10 μM 4'-chlorodiazepam (▨) in the presence and absence (set of bars on the left) of PK 11195. The concentration of PK 11195 is expressed as the log of the molar concentration.

GABA response elicited in the 293 cell line transfected with α_1 and β_1 subunits, and we failed to detect any change in the GABA-evoked Cl^- current (Fig. 6). Conversely, pressure application of both 4'-chlorodiazepam and DMCM on $\alpha_1,\beta_1,\gamma_2$-transfected cells produced a reduction in the GABA-elicited Cl^- current. These results are summarized in Table 1 where the observed decrease of GABA-activated Cl^- currents by picrotoxin is also compared with the lack of inhibition by 4'-chlorodiazepam and DMCM on the $\alpha_1\beta_1$ $GABA_A$ receptor. The GABA-regulated Cl^- currents of $\alpha_1\gamma_2$ or $\beta_1\gamma_2$ receptors showed a similar though lower inhibition by 4'-chlorodiazepam and DMCM. Flumazenil (10 μM) failed to affect the inhibition of 4'-chlorodiazepam (10 μM) on GABA-activated Cl^- currents in cells transfected with α_1, β_1, and γ_2 subunits ($n = 5$ cells), as observed in native receptors studied in cortical neurons. Flumazenil counteracted the DMCM-mediated inhibition of Cl^- currents in $\alpha_1\beta_1\gamma_2$ $GABA_A$ receptors (12), as reported in native receptors.

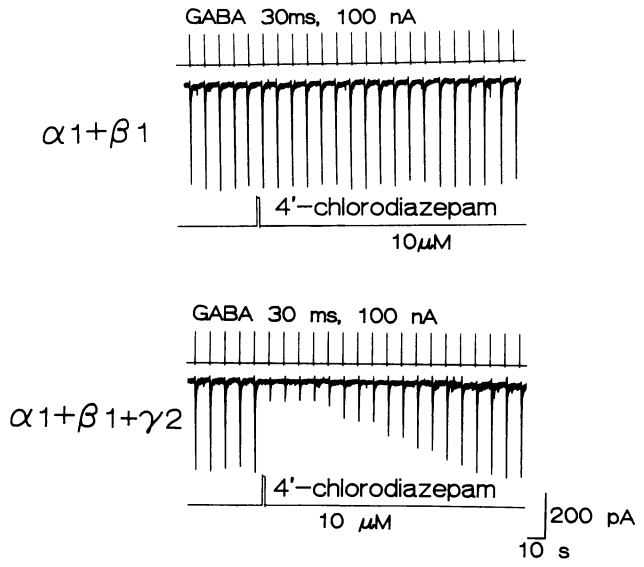

FIG. 6. Recording of GABA-evoked Cl^- currents in α_1- and β_1-transfected (*A*) or in $\alpha_1\beta_1\gamma_2$-transfected (*B*) mammalian cells. 4'-Chlorodiazepam does not show any action on the α_1- and β_1-transfected cells but reduces the Cl^- current when the γ_2 subunit is present. The cells were held at −40 mV.

Table 1. Inhibition of GABA-elicited Cl^- currents by 4'-chlorodiazepam and DMCM on various molecular forms of transiently expressed $GABA_A$ receptors

	4'-Chlorodiazepam			DMCM			PTX		
Subunits	% red	SD	*n*	% red	SD	*n*	% red	SD	*n*
$\alpha_1\beta_1$	0	—	9	0	—	3	42	12	4
$\alpha_1\gamma_2$	37	20	3	16	8	3	—	—	—
$\beta_1\gamma_2$	64	12	8	57	8	4	—	—	—
$\alpha_1\beta_1\gamma_2$	54	8	8	52	5	3	58	4	3

4'-Chlorodiazepam was at 10 μM; DMCM, 10 μM; and picrotoxin (PTX), 20 μM. The % red is the percentage reduction of GABA-activated Cl^- currents after pressure application of the modulator. *n*, Number of cells tested.

DISCUSSION

In rodents, 4'-chlorodiazepam causes convulsions and depresses $GABA_A$ receptor function (22). Our experiments confirm the reports by Skerritt *et al.* (2) and McEachern and Berg (8) that in primary neuronal culture, 4'-chlorodiazepam reduces GABA-activated Cl^- currents. Similarly to DMCM, 4'-chlorodiazepam also causes a voltage-independent inhibition of GABA-activated Cl^- currents. This supports the contention that DMCM and 4'-chlorodiazepam may not act by binding inside the chloride channel.

Since 4'-chlorodiazepam and DMCM effects appear to be similar, we wondered whether the two drugs act on the same receptor or possibly the same site. However, the affinity of 4'-chlorodiazepam is lower than that of DMCM, and the action of DMCM lasts longer than that of 4'-chlorodiazepam. The 4'-chlorodiazepam resistance and the DMCM sensitivity to flumazenil inhibition indicate that they do not act on the same site.

The resistance of 4'-chlorodiazepam action to flumazenil antagonism (2, 7) and its low affinity for TBPS (*t*-butylcyclophosphorothionate) binding sites (4) might indicate that 4'-chlorodiazepam does not act directly on the $GABA_A$ receptor. This consideration and the evidence that 4'-chlorodiazepam has a nanomolar affinity for the peripheral benzodiazepine receptors (23) might suggest that the action of this drug may even be unrelated to a binding site on $GABA_A$ receptors. However, despite the antagonism of 4'-chlorodiazepam-induced convulsions by the peripheral benzodiazepine antagonist PK 11195 (17), we were not able to inhibit the negative allosteric modulation of GABA-elicited Cl^- currents with PK 11195. These results might support the contention that 4'-chlorodiazepam acts isosterically at the picrotoxin binding site since it displaces TBPS.

To resolve these alternatives, we decided to study the action of 4'-chlorodiazepam in various molecular forms of $GABA_A$ receptors transiently expressed in cultured cells (12, 13). The picrotoxin-induced reduction of Cl^- currents elicited by GABA can be seen in *Xenopus laevis* oocytes injected with mRNA coding for α_1 and β_1 subunits of the $GABA_A$ receptor (11) and also is observed in the transient transfection system used by us (13). However, the Cl^- current generated by GABA in cells expressing α_1 and β_1 subunits is not inhibited by either DMCM (12) or 4'-chlorodiazepam (Table 1).

The difference in the action of picrotoxin, DMCM, and 4'-chlorodiazepam clearly suggests that the two allosteric negative modulators do not act by mimicking picrotoxin. Moreover, all of these compounds act in cells expressing the $GABA_A$ receptors assembled from $\alpha_1\beta_1$ and γ_2 subunits. That this transfection was successful is demonstrated by the

pharmacological profile of the modulation of GABA-evoked Cl^- currents and by the presence of [^{3}H]flumazenil and 4′-[^{3}H]chlorodiazepam binding [E. Slobodyansky in this laboratory (Georgetown University), unpublished results] in the transfected cells.

The action of DMCM and 4′-chlorodiazepam was also observed in cells expressing $\beta_1\gamma_2$ GABA receptors and, to a lesser extent, in cells expressing $\alpha_1\gamma_2$ receptors. Hence, our results are consistent with the view that 4′-chlorodiazepam inhibition requires the presence of the γ_2 subunit of $GABA_A$ receptors. We cannot conclude that the binding of either molecule is located in this subunit because expression of the γ_2 subunit alone generates GABA-regulated ion channels that are insensitive to DMCM and require high concentrations of GABA to elicit Cl^- currents (13). In the absence of such evidence, we suggest that the presence of the γ_2 subunit in $GABA_A$ receptor cooperatively allows the binding of 4′-chlorodiazepam, possibly to the benzodiazepine site on the α subunit, which can be photoaffinity labeled by flunitrazepam (24).

Our data suggests that the sites for 4′-chlorodiazepam and DMCM inhibition are not identical because of the marked difference in their sensitivity to flumazenil inhibition. Moreover, the coexpression of the γ_2 subunit seems to allow for the optimal action of 4′-chlorodiazepam because the native receptor in rodent neonatal neurons displays the same preference with regard to the flumazenil inhibition of DMCM but not 4′-chlorodiazepam action. However, in neonatal and adult neuron, the receptor characteristics may differ. Hence, it is important to confirm the selective inhibition of flumazenil in experiments using voltage-clamped adult neurons in brain slices.

Recently, a large variety of different structural subunits of the $GABA_A$ receptor have been cloned (12, 14, 25). This diversity suggests that certain combinations of subunits produce benzodiazepine-sensitive receptors, others might generate receptors modulated by 4′-chlorodiazepam, and yet others may contain both modulatory sites. The potentially differential expression of these receptor subunits in various central nervous system regions could have important applications in pharmacology (25). Hence, drugs may be found that selectively act on a $GABA_A$ receptor type in a given brain area, leaving unchanged the function of other subtypes expressed in the same area. This hypothesis is in agreement with autoradiographic studies (10) that show good, but not complete, correlation between the low-affinity 4′-chlorodiazepam binding sites and central benzodiazepine receptor densities. The discrepancy between the sedative and convulsant effects of 4′-chlorodiazepam might, for instance, derive from the presence of various subtypes of $GABA_A$ receptors.

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