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Substituted Pyrazinones, a New Class of Allosteric Modulators for γ-Aminobutyric Acid$_A$ Receptors

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SUMMARY

We discovered substituted pyrazinones as a new class of allosteric modulators of γ-aminobutyric acid (GABA)$_A$ receptors. Prototype pyrazinones, U-92813 [1-(furfuryl)-3,5-dichloro-6-phenylpyrazinone] and U-94863 [1-benzyl-3,5-dichloro-6-(2-chlorophenyl)pyrazinone], potentiated GABA-mediated Cl^- currents in cloned GABA$_A$ receptors with certain subtype selectivity. The drugs markedly enhanced the GABA response in the α1β2γ2 and α1β2 subtypes but not in the α1γ2 and β2γ2 subtypes expressed in human kidney cells. The dose-response profile of U-94863 in the α1β2 subtype was largely indistinguishable from that in the α1β2γ2 subtype, suggesting no critical role for the γ2 subunit in potentiation of the GABA response by the pyrazinones. The drugs also potentiated the GABA response in the α3β2γ2 and α6β2γ2 subtypes, indicating their nonselectivity toward the α isotypes. With respect to subtype selectivity, the pyrazinones differ not only from ligands for benzodiazepine receptors, which interact only with the subtypes containing αβγ subunits, but also from barbiturates and neurosteroids, which interact with all the subtypes tested in this study. The unique binding site for U-92813 on GABA$_A$ receptors was confirmed by the insensitivity of its action to Ro 15-1788, a classical benzodiazepine antagonist, and by the additive nature of its agonistic activity with that of barbiturates and neurosteroids. With respect to the mechanism of potentiation, the pyrazinones are similar to the other allosteric modulators, in that they potentiate the GABA response more effectively at low GABA concentrations than at high GABA concentrations. We propose that substituted pyrazinones represent a novel class of allosteric modulators of GABA$_A$ receptors, with their binding site probably located between the α and β subunits.

GABA, an inhibitory neurotransmitter, interacts with the GABA$_A$ receptor-Cl^- channel complex and induces synaptic membrane chloride conductances (1-5). The GABA-induced Cl^- conductances are allosterically modulated by three major types of drugs, benzodiazepines, barbiturates, and neurosteroids (3, 6-12). These drugs are therapeutically useful as anxiolytic, hypnotic, anticonvulsant, and anesthetic agents, to name a few, but display various undesirable side effects. Novel modulators of GABA$_A$ receptors with fewer side effects appear to be in demand. The search for such drugs is facilitated by the recent cloning of multiple subunits of GABA$_A$ receptors (13-16) and their stable expression in mammalian cells (17). Predominant subunits in the brain are α, β, and γ polypeptides of 48-55 kDa, each of which has several isotypes (13-18). Among many possible combinations of GABA$_A$ receptor subunits, the α1β2γ2 subtype, when expressed in human kidney cells (A293 cells), accommodates all known allosteric ligands for GABA$_A$ receptors (13-16, 19-22) and displays functional characteristics similar to those of neuronal GABA$_A$ receptors. Using this subtype, we screened chemicals of diverse structures for their ability to affect GABA-mediated Cl^- currents. From the screen, we discovered two substituted pyrazinones, U-92813 [1-(furfuryl)-3,5-dichloro-6-phenylpyrazinone] and U-94863 [1-benzyl-3,5-dichloro-6-(2-chlorophenyl)pyrazinone] (Fig. 1), as a new class of allosteric modulators and report here the characterization of their modes of interactions with several subtypes of GABA$_A$ receptors.

Materials and Methods

The stable cell lines expressing the indicated combinations of α1 (23), α3 (14), α6 (24), β2 (25), and γ2 (26) subunits of GABA$_A$ receptors were derived by transfection of plasmids containing cDNA and a plasmid encoding G418 resistance into A293 cells (17, 27). After 2 weeks of selection in 1 mg/ml G418, resistant cells were assayed for the ability to synthesize GABA$_A$ receptor mRNA, by Northern blotting. Positive cells were used for electrophysiology to measure GABA-induced Cl^- currents.

The whole-cell configuration of the patch-clamp technique (28) was used to record the GABA-mediated Cl^- currents in human embryonic kidney cells (A293) expressing various combinations of GABA$_A$ receptor subunits, as described earlier (29). Briefly, patch pipettes were prepared from borosilicate glass tubes and were fire-polished to a tip resistance of 0.5-2 MΩ when filled with a solution containing (in mM)

ABBREVIATIONS: GABA, γ-aminobutyric acid; 5α-THDOC, 3α,21-dihydroxy-5α-pregnan-20-one; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

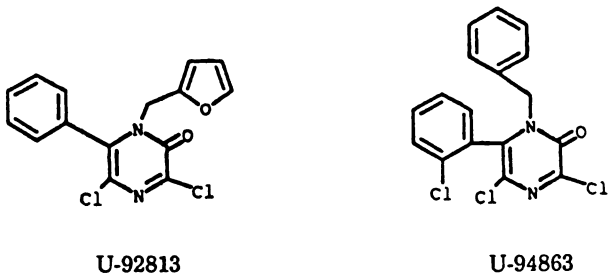

Fig. 1. Chemical structures of U-92813 and U-94863.

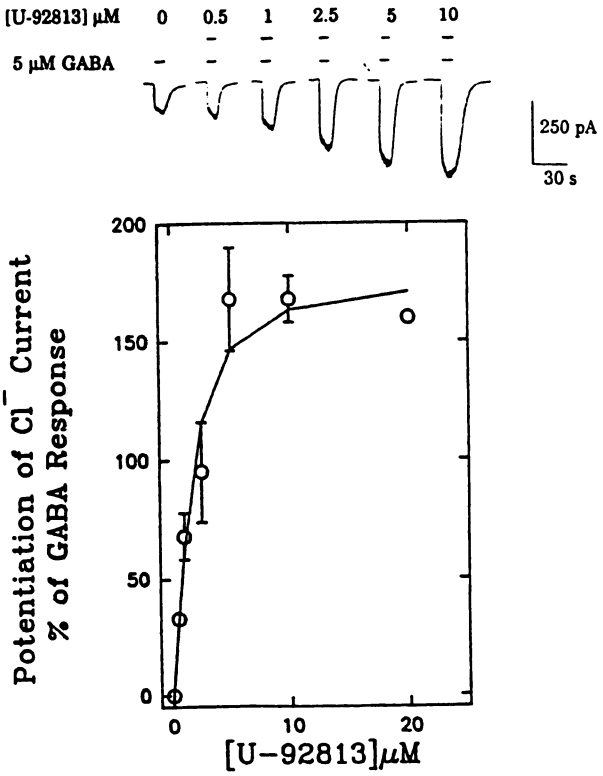

Fig. 2. Dose-response profile for potentiation of GABA-mediated Cl$^-$ currents by U-92813 with the $\alpha1\beta2\gamma2$ subtype of GABA$_A$ receptor expressed in A293 cells. The Cl$^-$ current was measured in the whole-cell configuration of the patch-clamp technique, at a holding potential of −60 mV, in the presence of a symmetric Cl$^-$ gradient. GABA (5 μM) without or with U-92813 at the indicated concentrations was applied for about 10 sec. The net increase in the amplitude of the current due to the presence of the drug was normalized to the response to GABA at 5 μM and plotted as a function of the drug concentration. *Solid line*, fitting of the data to the logistic equation given in the text. The data represent the mean ± standard error from four measurements (lower panel).

140 CsCl, 11 EGTA, 4 $MgCl_2$, 2 ATP, and 10 HEPES, pH 7.3. Cells were bathed in an external solution containing (in mM) 135 NaCl, 5 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, and 5 HEPES, pH 7.2. GABA and drugs were dissolved in the external solution to a final concentration of 5 μM, unless indicated otherwise, and were applied through a U-tube placed within 100 μm of the target cell. The current was recorded with an Axopatch 1D amplifier and a CV-4 headstage (Axon Instrument Co.). A Bh-1 bath headstage was used to compensate for changes in bath potentials. The currents were recorded with a Gould 220 recorder. GABA currents were measured at a holding potential of −60 mV at room temperature (21–24°).

Results

Interaction of substituted pyrazinones with various subtypes of GABA$_A$ receptors. We tested the ability of U-92813 to affect GABA-mediated Cl$^-$ currents in A293 cells

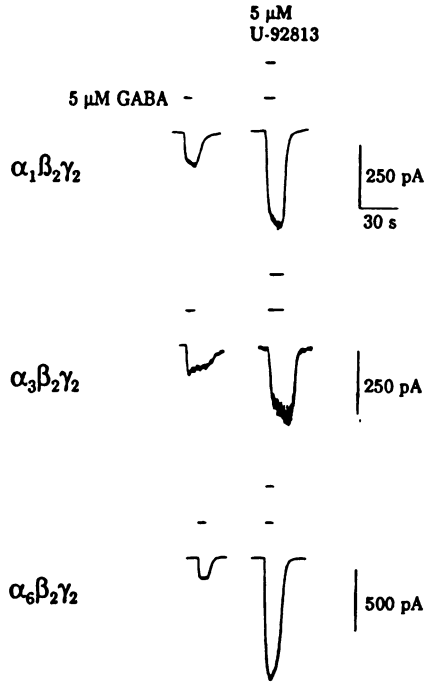

Fig. 3. Comparison of U-92813 action on the cloned GABA$_A$ receptors produced from the combination of $\alpha1$, $\alpha3$, or $\alpha6$ with $\beta2$ and $\gamma2$ subunits. GABA (5 μM) without or with U-92813 (5 μM) was applied to A293 cells expressing the $\alpha1\beta2\gamma2$, $\alpha3\beta2\gamma2$, or $\alpha6\beta2\gamma2$ subtypes, after the whole-cell configuration of the patch-clamp technique had been established. The drug potentiated GABA-induced Cl$^-$ currents in the three subtypes of GABA$_A$ receptors. The degree of the potentiation by U-92813 ranged from 130 to 200% with the $\alpha1\beta2\gamma2$ and $\alpha3\beta2\gamma2$ subtypes but reached >300% with the $\alpha6\beta2\gamma2$ subtype.

expressing diverse subtypes of GABA$_A$ receptors, by using the whole-cell configuration of the patch-clamp technique. Fig. 1 shows the effect of U-92813 at various concentrations (0.5–10 μM) on GABA-induced Cl$^-$ currents with the $\alpha1\beta2\gamma2$ subtype. U-92813 induced no current without GABA (data not shown) but dose-dependently potentiated GABA (5 μM)-mediated Cl$^-$ currents. The dose-response curve for U-92813 (Fig. 2) was analyzed using a logistic equation (30), $E = E_m \times [\text{drug}]^n/(K_d^n + [\text{drug}]^n)$, where E is the degree of potentiation by a drug at a given concentration ([drug]), E_m is the maximal effect, n is a slope factor, and K_d is the concentration of drug needed to produce 50% of the maximal effect. From the analysis we obtained a K_d of 1.4 ± 0.4 μM, E_m of 176 ± 18% (as normalized to 5 μM GABA response), and n of 1.1 ± 0.4 for U-92813. In the same batch of cells, diazepam (2 μM), a classical benzodiazepine agonist, potentiated the GABA response maximally by 95 ± 20%.

The potentiating effect of U-92813 was not blocked by Ro 15–1788 at a concentration 5 times greater than that of U-92813 (data not shown). U-92813 also potentiated GABA-induced Cl$^-$ currents in the $\alpha3\beta2\gamma2$ and $\alpha6\beta2\gamma2$ subtypes (Fig. 3); the drug (5 μM) enhanced the GABA (5 μM) response by 180 ± 24% in the $\alpha3\beta2\gamma2$ subtype and by 350 ± 47% in the $\alpha6\beta2\gamma2$ subtype.

Potential interactions of U-92813 with pentobarbital and 5α-THDOC were examined in the $\alpha1\beta2\gamma2$ and $\alpha6\beta2\gamma2$ subtypes, two receptors with distinctive pharmacology (24). As shown in Fig. 4, the potentiation of GABA-induced Cl$^-$ currents by U-92813 at 5 μM (a nearly saturating dose) was additive with that of pentobarbital (5 μM) and 5α-THDOC (20 nM) for both

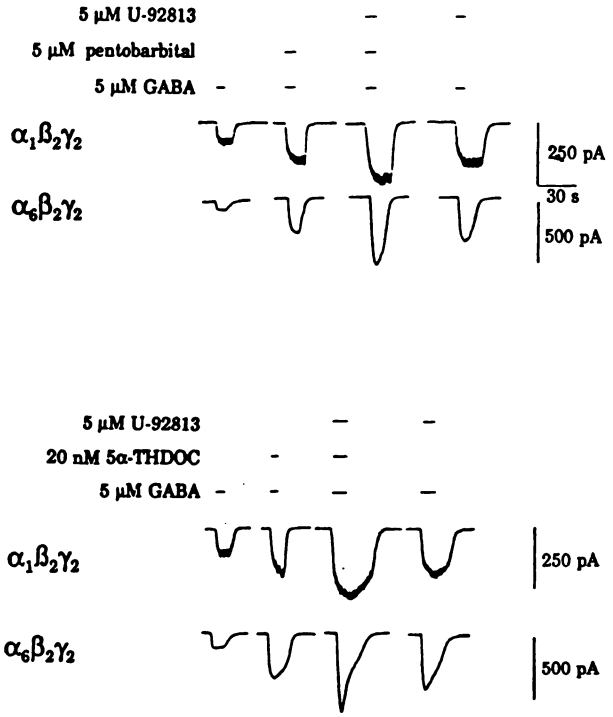

Fig. 4. Nonadditivity of the action of U-92813 with that of pentobarbital or 5α-THDOC. Cl^- currents were induced with GABA (5 μM) alone or in various combinations with U-92813 (5 μM), pentobarbital (5 μM), and/or 5α-THDOC (20 nM) in A293 cells expressing the α1β2γ2 or α6β2γ2 subtypes of $GABA_A$ receptors. With the α6β2γ2 subtype, the combination of the two drugs and GABA seemed to induce desensitization and therefore GABA was applied last, although it is shown here between the traces for the two drugs. Potentiation of the GABA response by pentobarbital and 5α-THDOC, a neurosteroid, was additive with a near-maximal potentiation by U-92813 in the two subtypes of $GABA_A$ receptors expressed in A293 cells.

subtypes. With the α1β2γ2 subtype, the GABA-induced current increased by 136% in the presence of U-92813 (5 μM), by 118% in the presence of pentobarbital (5 μM), and by 245% in the presence of the two together. Similarly, the degree of potentiation of GABA-induced Cl^- currents by the mixture of U-92813 and 5α-THDOC (198%) reached the combined level of potentiation by each drug, 110 and 86%, respectively. Potentiation of GABA-induced Cl^- currents by U-92813 in the α6β2γ2 subtype was about 2 times greater than that in the α1β2γ2 subtype, reaching 363 and 275% in two separate patches, and its effect was still additive with that of pentobarbital (270%) and 5α-THDOC (188%).

We further examined the effect of U-92813 on GABA-mediated Cl^- currents with subtypes made of two $GABA_A$ receptor subunits, i.e., α1β2, α1γ2, and β2γ2 (Fig. 5). The drug potentiated the GABA (5 μM) response by 154 ± 26% in the α1β2 subtype but had no effect in the α1γ2 and β2γ2 subtypes. This selective action by U-92813 differed from nonselective potentiation by pentobarbital (10 μM) and 5α-THDOC (20 nM) with these subtypes; the latter two potentiated GABA-induced Cl^- currents with all the subtypes we tested, including α1γ2 and β2γ2 (data not shown). This subtype selectivity of U-92813 could be extended to other compounds in the 2-pyrazinone series. For instance, U-94863 potentiated GABA-induced Cl^- currents only with the subtypes containing both α1 and β2 subunits, and its dose-response profile in the α1β2 subtypes

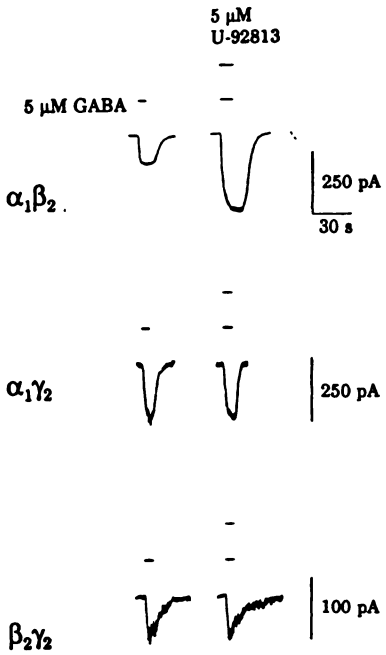

Fig. 5. Comparison of U-92813 action with the α1β2, α1γ2, or β2γ2 subtypes of $GABA_A$ receptors expressed in A293 cells. Cl^- current was induced with GABA (5 μM) alone or with U-92813 (5 μM) in the whole-cell mode of the patch-clamp technique. U-92183 potentiated the GABA response with the α1β2 subtype to the same extent (160%) as with the α1β2γ2 subtype but did not affect the responses with the α1γ2 and β2γ2 subtypes. It should be noted that pentobarbital (5 μM) and 5α-THDOC (20 nM) increased the GABA response by >150% with all three subtypes.

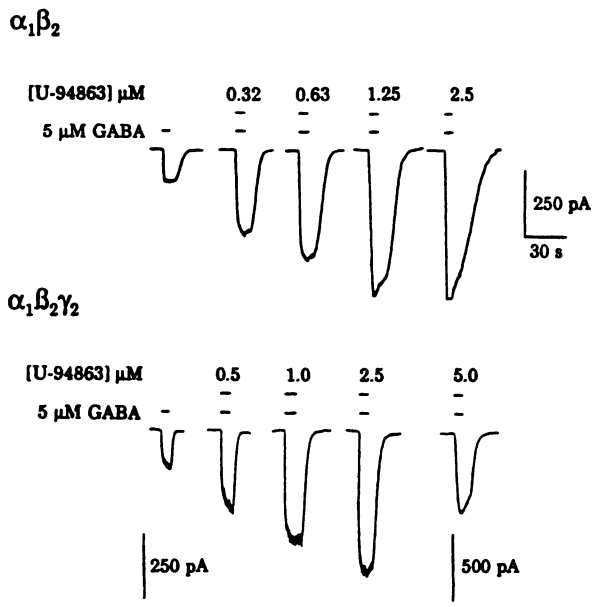

Fig. 6. Comparison of dose-response profiles for U-94863 action with the α1β2 and α1β2γ2 subtypes of $GABA_A$ receptors. GABA (5 μM) alone or with U-94863 at the indicated concentrations was applied to cells expressing the α1β2 and α1β2γ2 subtypes, after the whole-cell mode of the patch-clamp technique had been established. The dose-response profiles were similar in the two subtypes (see text).

was quite similar to that in the α1β2γ2 subtype (Fig. 6). From analysis of the data using the equation given above, we obtained a $K_{0.5}$ of 0.35 ± 0.2 μM, n of 1.1 ± 0.1, and E_m of 329 ± 16% for U-94863 with the α1β2 subtype, with corresponding values of

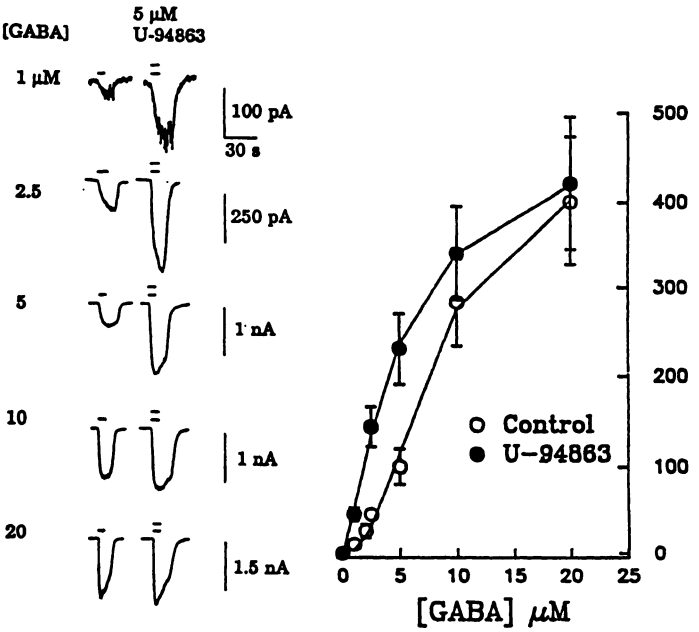

Fig. 7. Dependency of potentiation of GABA-induced Cl^- currents by U-94863 on the concentration of GABA. Cl^- current was induced with GABA at the indicated concentration, with or without U-94863 (5 μM), in A293 cells expressing the $\alpha1\beta2\gamma2$ subtype of $GABA_A$ receptors. The amplitude of peak Cl^- currents at various GABA concentrations was normalized to that observed with GABA at 5 μM and was plotted as a function of GABA concentration. The data are presented as means ± standard errors from three experiments. *Lines*, fitting of the data to the logistic equation given in the text. The degree of potentiation by U-94863 progressively decreased as the concentration of GABA was raised (*right panel*) (see text).

0.87 ± 0.2 μM, 1.2 ± 0.2, and 321 ± 26% for the $\alpha1\beta2\gamma2$ subtype. The similarity in these parameters further supports the notion that the 2-pyrazinones interact with the $\alpha1\beta2$ and $\alpha1\beta2\gamma2$ subtypes in the same manner.

Dependence of the agonistic activity of substituted pyrazinones on GABA concentration. U-94863 differentially potentiated Cl^- currents in the $\alpha1\beta2\gamma2$ subtype, depending on the GABA concentration (Fig. 7). Cl^- currents observed with GABA at 1 μM were enhanced by 420 ± 30% in the presence of U-94863 (5 μM). As the concentration of GABA was raised, the degree of potentiation of Cl^- currents by U-94863 progressively decreased, i.e., <20% in the presence of GABA at 10 μM. From GABA dose-response curves, we found that U-94863 (5 μM) decreased the half-maximal concentration for GABA from 8.4 ± 0.8 to 5.6 ± 0.6 μM and the slope factor from 2.2 ± 0.2 to 1.2 ± 0.1, with little effect on the maximal level of stimulation (465 ± 32 versus 504 ± 26).

Discussion

The major point of this study is a discovery of substituted pyrazinones as a novel class of allosteric modulators for $GABA_A$ receptors; their binding site appears to be different from those for benzodiazepines, barbiturates, and neurosteroids. This is based on the following observations made in this study. 1) Potentiation of GABA-induced Cl^- currents by the 2-pyrazinones is insensitive to Ro 15–1788, a classical benzodiazepine antagonist, and is observed with the subtypes of $GABA_A$ receptors that are known to have no benzodiazepine sites, such as the $\alpha1\beta2$ subtype (22). 2) Potentiation of GABA-mediated Cl^- currents by pentobarbital or 5α-THDOC is additive with the maximal response of U-92813. 3) The 2-pyrazinones potentiate the GABA response only in the subtypes containing both $\alpha1$ and $\beta2$ subunits, whereas pentobarbital and neurosteroids interact in all the subtypes we tested, including the $\alpha1\gamma2$ and $\beta2\gamma2$ subtypes.

This subtype selectivity of the 2-pyrazinones provides two possibilities for localization of their binding site(s) on $GABA_A$ receptors; one is the boundary between the $\alpha1$ and $\beta2$ subunits and the other is on either one of the two subunits but created by quaternary interactions of the two subunits. The former is consistent with the notion that allosteric modulators interact at subunit interfaces in oligomeric protein complexes (31). The latter could not be ruled out at present but is of low probability, judging by the fact that several different combinations of $GABA_A$ receptor subunits produced no qualitative differences in binding patterns of primary ligands such as GABA and *t*-butylbicyclophosphorothionate,[1] although their binding regions may not necessarily reflect all the conformational changes produced by quaternary interactions of subunits.

We found that potentiation of GABA-induced Cl^- currents by the 2-pyrazinones (U-94863) was highly sensitive to the concentration of GABA; the degree of potentiation by the drug progressively decreased as the GABA concentration was raised (see Fig. 7). The marked potentiation of Cl^- currents by the drug, particularly at low concentrations of GABA, apparently changed the dose-response curve from sigmoidal to hyperbolic shape (changing the slope factor from 2.2 to 1.2). It is not clear how the decrease in the slope factor is related to changes in interactions of GABA with the receptors. It may not mean, however, that channel openings are induced by occupancy of one rather than two or more GABA binding sites, because the half-maximal concentration for GABA action on Cl^- currents in the presence of U-94863 was still about 2 orders of magnitude greater than the dissociation constant for the high affinity GABA site. An earlier study reported that chlordiazepoxide, a benzodiazepine, also produces a similar decrease in the slope factor for GABA-mediated influx of ^{36}Cl in rat brain membranes (32). Future single-channel studies on $GABA_A$ receptors in the presence of U-94863 or chlordiazepoxide could provide more information about interactions of GABA with the receptors. In any event, U-94863 possesses the ability to make GABA (particularly at low concentrations) more efficient in inducing channel openings, like benzodiazepines and the imidazoquinoxaline series (32, 33).

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