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Characterization of Functional Interactions of Imidazoquinoxaline Derivatives with Benzodiazepine-γ-Aminobutyric Acid$_A$ Receptors

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SUMMARY

U-78875 [imidazo[1,5-a]quinoxalin-4(5*H*)-one, 3-(5-cyclopropyl-1,2,4-oxadiazol-3-yl)-5-(1-methylethyl)] belongs to a series of imidazoquinoxaline derivatives, recently discovered ligands with high affinity for benzodiazepine receptors. In this study, we have examined the drug and its analogs for their modes of interaction with the receptors, with a particular emphasis on finding molecular determinants for their functional properties. Changes in the substituents on N_5 and C_6 of the heterocyclic ring produced no major effects on binding characteristics but yielded drugs of widely varying efficacy (antagonist to full agonist), measured as γ-aminobutyric acid (GABA)-mediated $^{36}Cl^-$ uptake and *t*-butylbicyclophosphoro[^{35}S]thionate binding in rat cerebrocortical membranes. The relative binding affinity and efficacy of the analogs measured in brain membranes were similar to those in cloned GABA$_A$ receptors of the $\alpha_1\beta_2\gamma_2$ (type I) and $\alpha_3\beta_2\gamma_2$ (type II) subtypes. The imidazoquinoxalines showed no marked subtype selectivity. Their K_i value against [^{3}H]flunitrazepam binding for type I was only 2–3 times lower than that for type II, and their rank order for agonistic activity was the same in the two subtypes, measured as GABA-mediated Cl^- currents in human kidney cells (A293) expressing the subtypes of GABA$_A$ receptors. According to computational modeling of the drugs using both molecular and quantum mechanics, the agonistic activity of the imidazoquinoxaline derivatives depends on the presence of a bulky alkyl substituent at N_5 and the deformation of the substituted portion of the otherwise planar ring system induced by a bulky moiety at N_5 or C_6. With a fixed N_5 substituent (isopropyl), the relative efficacy in the brain membranes, as well as in the cloned receptors, appeared to be dependent on the degree of the ring deformation. This out-of-plane portion of the imidazoquinoxalines can be assigned to the general region occupied by the 5-phenyl group of diazepam and other agonistic functional groups of several nonbenzodiazepine ligands. It seems that this region, apparently common to various agonistic ligands, interacts with an agonistic pocket in type I and type II subtypes of the benzodiazepine receptors in the brain. Our results also provide direct support for the view that the agonists and nonagonists share largely overlapping binding regions in the benzodiazepine receptor, which has been proposed earlier from *in vivo* efficacy measurements of other series of ligands.

The benzodiazepine binding region on the GABA$_A$ receptor/Cl^- ionophore accommodates not only classical benzodiazepines but also chemical agents of diverse structures (1–5). Considerable efforts have focused on discovering features of various ligands that are essential for their interaction with GABA$_A$ receptors (6–15). The common method has been the superpositioning of the X-ray crystallographic or computational three-dimensional structures of various ligands. Generally, two very different working hypotheses have been used in approaching the problem. One approach assumes that the inverse agonists and/or antagonists bind to sites independent of the agonist site (6, 9, 11), whereas the other seeks to get maximum structural similarity among the agonists, antagonists, and inverse agonists, assuming their largely overlapping recognition sites (8, 10–12, 14). In the case of agonists, a number of proposals relevant to both hypotheses identify two electron-rich heteroatoms, an aromatic ring, and their approximate coplanarity and proper geometry as the key recognition modulators (6, 8, 11, 12, 14). Diverse proposals have been advanced, however, for the recognition and functionality of nonagonists and their relations to agonists (6, 8, 9, 11, 12, 14, 16–18). The proposed key modulators for nonagonists include the presence of an auxiliary proton-accepting group and its relative geometry with respect to other recognition modulators (8), the absence of a freely rotating aromatic ring or other bulky group in an OPR thought to be essential for agonist activity (14), or the different localization of the antagonist binding sites between those for the agonist and the inverse agonist inside the benzodiazepine binding sites (9). In a more recent paper, Hollishead *et al.* (11) reported that minor changes in the side chains of β-

ABBREVIATIONS: GABA, γ-aminobutyric acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; OPR, out-of-plane region; MEP, molecular electrostatic potential; TBPS, *t*-butylbicyclophosphorothionate.

carbolines produced inverse agonists, antagonists, and even agonists, judging from their anticonvulsant activity in mice, and they proposed that "the ligand-receptor interactions necessary for an agonist response are clearly different from those necessary for inverse agonist/antagonist activity, although a hydrogen bond site on one ligand class may overlap with that of the other class" (p. 1067). Villar *et al.* (12) have also recently examined a similar series of β-carbolines and pyrazoloquinolines but proposed the key structural feature for the ligand functionality as being the geometry of the most hydrophobic aromatic ring relative to the two most electron-rich heteroatoms. Apparently, the essential features for functionality of benzodiazepine ligands are not established.

The discrepancy among these proposals appears to arise not only from the complexity of the benzodiazepine receptor but also from two other sources. First, most substituents in these ligands leading to the functional variations are considerably removed from the major ring system and possess considerable conformational freedom, making their precise structure difficult to determine. Second, the structure-activity relationships used in most proposals were derived from *in vivo* measurements of anticonvulsant activity, which may differ from the inherent efficacy measured *in vitro*, free from metabolism and variations in pharmacokinetics. Furthermore, the anticonvulsant activity hardly represents possible differential actions of the drugs with multiple subtypes of GABA$_A$ receptors in the brain.

In this study, we have used a series of imidazoquinoxaline derivatives, ligands of high affinity for the benzodiazepine binding region on GABA$_A$ receptors, that induce anxiolytic and hypnotic actions in experimental animals (19). With the analogs of U-78875 (see Fig. 1), we found that minor variations in substituents on the fairly rigid planar ring system produced a spectrum of agents with varying functional properties (antagonist to full agonist), as measured by $^{36}Cl^-$ uptake in rat cerebrocortical synaptoneurosomes, [^{35}S]TBPS binding, or GABA-induced Cl^- currents in A293 cells expressing type I and type II GABA$_A$ receptors, consisting of $\alpha_1\beta_2\gamma_2$ and $\alpha_3\beta_2\gamma_2$ subunits, respectively (20). Here we characterize the functional and binding properties of the U-78875 series, using strictly *in vitro* assays, seek their key structural features by means of computational three-dimensional structural modeling, and compare the results with the proposed models of receptor modulation.

Materials and Methods

Computer modeling. Initially, the conformational properties of the imidazoquinoxaline ring system were studied with molecular mechanics methods, using the MACROMODEL implementation of the MM2 force field (21, 22). Calculations were performed on a number of analogs (see Table 1), in which R_1 and R_2 were varied and the cyclopropyl oxadiazole ring was replaced with hydrogen. Low energy conformers were determined by full energy minimization performed on two sets of initial conformers, generated by random (Monte Carlo) (22) and by systematic (23) searching of conformational space. All torsional angles (cyclic and acyclic) were varied in the initial search procedures. Both methods led to the same set of low energy conformers.

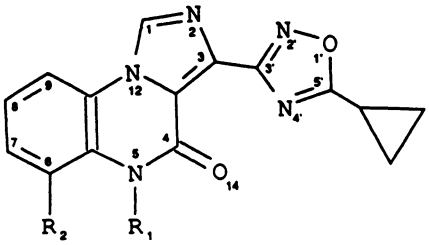

Fig. 1. Molecular structure and atom numbering of U-78875 analogs.

Subsequently, semiempirical quantum mechanical calculations were carried out on the full molecular structures of three representative analogs, U-78875, U-79098, and U-82249, using the MOPAC program with the AM1 parameter set (24). Geometry optimizations were performed using a number of initial conformations based on molecular mechanics studies with selected orientations of the cyclopropyl oxadiazole substituent, in order to determine the global minimum energy conformation of each molecule. In addition, the MEP at points on a van der Waals surface was computed from the resultant electronic wavefunctions.

Cloned GABA$_A$ receptors. The stable cell lines expressing the indicated combinations of α_1 (25), α_3 (26), β_2 (27), and γ_2 (28) subunits of GABA$_A$ receptors were derived by transfection of plasmids containing cDNA and a plasmid encoding a G418 resistance gene into the A293 cells (29). After 2 weeks of selection in 1 mg/ml G418, resistant cells were assayed for the ability to synthesize all three GABA$_A$ receptor mRNAs, by Northern blotting. Positive cells were used for electrophysiology, to measure GABA-induced Cl^- currents.

For binding studies, because of relatively low levels of expression of the GABA$_A$ receptor subtypes in A293 cells, we used the baculovirus system for receptor expression in SF-9 cells, an insect ovary cell line, where a high level of expression of ion channels has already been shown (30). We showed earlier that the infected insect cells expressed GABA-mediated Cl^- currents, as well as benzodiazepine binding sites having the same pharmacological specificity as animal cells expressing the same receptor subunits (31). For instance, zolpidem, an imidazopyridine, displayed a K_i value of 18.7 ± 0.6 nM for the $\alpha_1\beta_2\gamma_2$ subtype (type I), compared with a value of 286 ± 3 nM for the $\alpha_3\beta_2\gamma_2$ subtype (type II), confirming its selectivity toward type I, as in mammalian cells.

Membrane preparations and binding measurements. Briefly, SF-9 cells infected with baculovirus carrying cDNAs for $\alpha_1\beta_2\gamma_2$ or $\alpha_3\beta_2\gamma_2$ subunits were harvested in 2-liter batches 60 hr after infection. The membranes were prepared in normal saline after homogenization with a Polytron PT 3000 (Brinkman) for 4 min. Unbroken cells and large nuclear aggregates were removed by centrifugation at 1000 × *g* for 10 min. The membranes were then recovered with a second centrifugation of the supernatant at 40,000 × *g* for 50 min. The membranes were resuspended to a final concentration of 5 mg/ml, in a solution containing 300 mM sucrose, 5 mM Tris·HCl, pH 7.5, and glycerol to a final concentration of 20%, and were stored at −80°.

Rat cerebrocortical synaptosomal membranes from brain cortices of male Sprague-Dawley rats (150–180 g) were prepared as described elsewhere (32). Binding of [^{3}H]flunitrazepam was measured in medium containing 6 nM radioactive ligand, 110 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, and 30 μg of membrane proteins, in a total volume of 500 μl. The reaction was carried out at 4° for 60 min. To obtain the IC_{50} values, we included competing nonradioactive drugs at six different concentrations and analyzed the data using a computer program for the binding isotherm. The K_i values were computed using eq. 1,

$$K_i = IC_{50}/(1 + [L]/K_d) \quad (1)$$

where [L] and K_d are, respectively, the concentration of [^{3}H]flunitrazepam and its dissociation constant, which was measured to be 1.2 ± 0.1 nM, from Scatchard analysis, in the rat cerebrocortical membranes.

Binding of [^{35}S]TBPS in the rat brain membranes was measured in medium containing 2 nM [^{35}S]TBPS, unless specified otherwise, 50 μg of membrane proteins, 1 M NaCl, and 10 mM Tris·HCl, pH 7.4, in a total volume of 500 μl. Drugs were added in concentrated methanolic solutions; the level of methanol did not exceed 0.2% and was maintained constant in all tubes. The mixtures were incubated for 120 min at 24°.

In all binding assays, the reaction mixtures were filtered over a

TABLE 1

Characterization of interactions between benzodiazepine sites on $GABA_A$ receptors and U-78875 analogs, and comparison of their apparent efficacy with the degree of imidazoquinoxaline ring deformation

The affinity of the U-78875 analogs for the benzodiazepine site was estimated from their ability to inhibit [^{3}H]flunitrazepam (6 nM) binding to rat cerebrocortical synaptosomal membranes. The IC_{50} values, obtained from dose-response curves consisting of six different concentrations, were converted to K_i values using eq. 1. Functional interaction of the drugs with the benzodiazepine site was monitored as their ability to inhibit [^{35}S]TBPS binding in the presence of 2 μM GABA and to potentiate 5 μM GABA-mediated $^{36}Cl^-$ uptake in rat cerebrocortical membranes. The effects of the drugs at 1 μM on TBPS binding and at 5 μM on $^{36}Cl^-$ uptake were normalized with respect to the corresponding actions of diazepam. The data represent the mean ± standard error of three to nine separate experiments. The various substituent-induced deformations in the imidazoquinoxaline ring were measured as the dihedral angle (θ) between the R_1-C_6-N_5 and C_6-N_5-R_2 planes, using molecular and quantum mechanics. The other experimental and computational details were described in Materials and Methods.

Compound	R_1	R_2	Binding K_i	[^{35}S]TBPS	$^{36}Cl^-$ uptake[a]	θ	
						MM2	AM1
			nM	% of diazepam	% of diazepam	deg	
U-78263	Methyl	H	7.5 ± 0.6	−7 ± 3	−14 ± 24	0	
U-78280	Ethyl	H	5.6 ± 0.4	−7 ± 2	−12 ± 16	±1.9	
U-78875	Isopropyl	H	2.8 ± 0.2	6 ± 4	4 ± 14	±16.3	±15.6
U-82249	*t*-Butyl	H	4.5 ± 0.3	121 ± 15	101 ± 17	±38.5	±32.3
U-79098	Isopropyl	Cl	1.4 ± 0.2	78 ± 9	103 ± 37	±51.6	±43.0
U-81139	Methyl	CF_3	0.7 ± 0.1	−10 ± 4	−3 ± 22	±49.3	
U-89320	Isopropyl	OCH_3	7.4 ± 0.7	64 ± 10	48 ± 14	±39.6	

[a] Rat cerebrocortical synaptosomal membranes.

Whatman GF/B filter under vacuum. The filters were washed three times with 4 ml of the respective reaction buffer without radioisotope and were counted for radioactivity. Nonspecific binding was estimated in the presence of 2 μM diazepam or 2 μM unlabeled TBPS and was subtracted, to compute specific binding.

$^{36}Cl^-$ uptake studies. Rat cerebrocortical synaptoneurosomes were prepared following the procedure of Hollingsworth *et al.* (33). $^{36}Cl^-$ uptake in the synaptoneurosomes was measured by a rapid filtration technique, using Whatman GF/B filters, as described elsewhere (34). A typical incubation medium contained 0.2 μCi/ml $Na^{36}Cl$, 118 mM NaCl, 5 mM KCl, 1.8 mM $MgSO_4$, and 20 mM HEPES/Tris, pH 7.0, with or without test drugs. Drugs were added in concentrated methanol solutions; the level of methanol did not exceed 0.4% and was maintained constant in all tubes. The membrane suspensions were preincubated for 5 min at 30°. The reaction was initiated by mixing of equal volumes (125 μl) of the membrane suspension (1 mg of protein) and the reaction mixture containing $^{36}Cl^-$, at 30°. After 5 sec, the reaction was terminated by addition of ice-cold NaCl incubation buffer. The mixture was filtered over a Whatman GF/B filter under vacuum, and the filters were washed four times with 5 ml of ice-cold NaCl incubation buffer. The radioactivity on the filters was counted in the presence of Instagel (Packard) (15 ml). The amount of protein in membranes was determined by the method of Lowry *et al.* (35), using bovine serum albumin for calibration.

Electrophysiology. The whole-cell configuration of the patch-clamp technique (36) was used to record the GABA-mediated Cl^- currents in the A293 cells expressing the $\alpha_1\beta_2\gamma_2$ or $\alpha_3\beta_2\gamma_2$ subtype, as described earlier (37). Briefly, patch pipettes made of borosilicate glass tubes were fire-polished and showed a tip resistance of 0.5–2 MΩ when filled with a solution containing (in mM) 140 CsCl, 11 EGTA, 4 $MgCl_2$, 2 ATP, and 10 HEPES, pH 7.3. The cell-bathing external solution contained (in mM) 135 NaCl, 5 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, and 5 HEPES, pH 7.2 (normal saline). GABA at the concentration of 5 μM in the external solution, with or without indicated drugs, was applied for 10 sec through a U-tube placed within 100 μm of the cells, unless indicated otherwise. 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 the holding potential of −60 mV at room temperature (21–24°).

Results

Molecular conformations. Molecular mechanics studies of the imidazoquinoxaline ring system showed that, with the exception of U-78263, each analog possesses a pair of degenerate mirror-image conformers at the global minimum energy. The mirror-image conformers differ structurally by equal but opposite deformations of the quinoxaline ring from a planar structure. A simple measure of the deformation is given for each analog in Table 1 by the angle θ, defined as the dihedral angle between the X-C_6-N_5 and the C_6-N_5-Y planes, where X and Y are the points of attachment of R_2 and R_1, respectively (see Fig. 1). The mirror-image isomers are distinguished by the sign of θ. In U-78263, the ring system was found to be planar; consequently, only a single conformer was found at the global minimum. The structures of a number of higher energy conformers were also determined for each analog. However, the calculated strain energies were generally found to be too large, relative to the global minimum energy, for such structures to be significantly populated.

The degree of ring deformation is clearly associated with steric interactions among the carbonyl, R_1, and R_2 substituents. For example, with R_2 fixed as H, the change in R_1 from methyl to ethyl, isopropyl, or *t*-butyl increased the magnitude of θ from 0° to 1.9°, 16.3°, and 38.5°, respectively. Furthermore, an R_2 substituent bulkier than H increased ring deformation; within pairs having the same R_1 group, Cl at R_2 increased θ from 16.3° (U-78875) to 51.6° (U-79098), CF_3 from 0° (U-78263) to 49.3° (U-81139), and OCH_3 from 16.3° (U-78875) to 39.6° (U-89320).

Fig. 2 shows an overlay of the ring systems of one of the low energy conformers of U-79098 (Cl substituent) and U-78875, obtained by least-squares superposition of all ring carbon and nitrogen atoms except C_6 and N_5. It is evident that the quinoxaline ring deformation primarily alters the relative spatial positions of the R_1 and R_2 groups, without major distortions to the rest of the molecular structure.

The characteristics of the minimum energy structures of U-78875, U-79098, and U-82249 obtained from AM1 quantum mechanical calculations were similar to those from the MM2 studies of the ring systems described above. Once again, a pair of mirror-image conformers was found at the global minimum energy. The AM1 calculations showed the same trend between ring deformation and substituent as the MM2 method but predicted slightly less ring deformation; the value of θ from the AM1 calculations was ±15.6°, ±32.3°, and ±43° for U-78875,

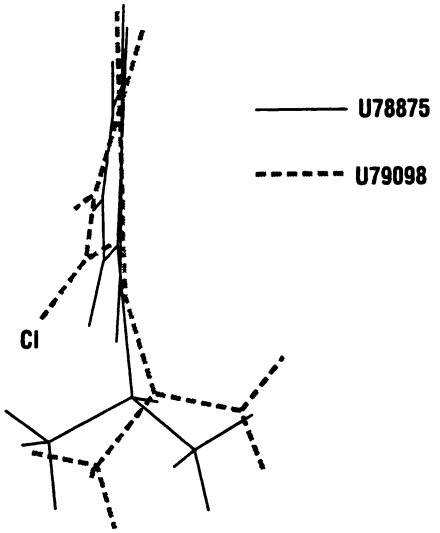

Fig. 2. Superposition of minimum energy structures of U-78875 and U-79098.

TABLE 2

Minimum values of the MEP near selected heteroatoms of U-78875, U-79098, and U-82249

Compound	MEP, Minimal					
	N_2	O_{14}	$N_{2'}$	$N_{4'}$	$O_{1'}$(u)*	$O_{1'}$(d)*
	kcal					
U-78875	−45.7	−55.7	−40.3	−52.2	−27.9	−27.0
U-79098	−43.8	−55.0	−38.7	−48.9	−24.5	−26.3
U-82249	−45.9	−53.5	−40.0	−50.4	−25.2	−27.4

* (u), up; (d), down.

U-82249, and U-79098, respectively. In the AM1-minimized structures, respective internuclear distances involving all atoms except those in R_1 and R_2 differed by no more than 0.05 Å in the three analogs. This indicates a high degree of structural similarity among the analogs in both the imidazoquinoxaline ring and the oxadiazole group. Thus, the principal differences in structure involve only the identity and relative orientations of R_1 and R_2.

Electronic properties. There is also a high degree of similarity in the electrostatic properties of U-78875, U-79098, and U-82249, particularly in key regions of possible hydrogen bonding sites. This is illustrated by the data in Table 2, which lists the MEP minima at points on a van der Waals surface that are nearest to atoms N_2, O_{14}, $N_{2'}$, $N_{4'}$, and $O_{1'}$. These points correspond approximately to "lone-pair" regions near N_2, $N_{2'}$, and $O_{1'}$ and to an electron-rich region between O_{14} and $N_{4'}$. The values of the MEP minima were not influenced by either ring deformation or the identity of R_1 or R_2. Moreover, respective distances between MEP minima were found to differ by no more than 0.1 Å in the three analogs.

Binding and functional properties of U-78875 and its analogs. U-78875 displaced [^{3}H]flunitrazepam binding in rat cerebrocortical synaptosomal membranes with a K_i value of 2.8 ± 0.2 nM (Fig. 3A; Table 1). Functionally, the drug showed little effect on GABA-induced $^{36}Cl^-$ uptake in rat cerebrocortical synaptoneurosomes (Table 1) but blocked the potentiating action of diazepam (at 5 μM) on $^{36}Cl^-$ uptake, with a half-maximal inhibitory dose of 1.2 μM (Fig. 3B). Typically, diazepam at 5 μM increased GABA-mediated Cl^- uptake by 5.2 ± 0.8 nmol/mg of protein/5 sec. This shows qualitatively the

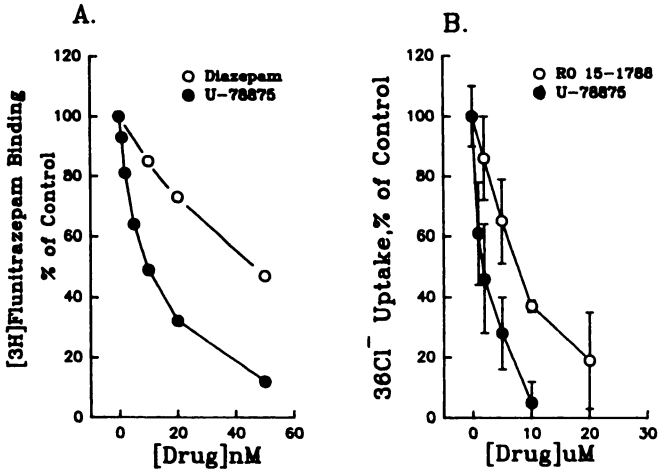

Fig. 3. Plots showing interactions of U-78875 with the benzodiazepine sites on GABA$_A$ receptors. A, The equilibrium binding of [^{3}H]flunitrazepam to rat cerebrocortical synaptosomal membranes was dose-dependently inhibited by U-78875 and diazepam. The control represents the level of radioligand binding in the absence of drugs. B, Diazepam (5 μM) potentiated GABA (5 μM)-mediated $^{36}Cl^-$ uptake by 5.2 ± 0.8 nmol/mg of protein/5 sec (100%) in rat cerebrocortical synaptoneurosomes (control). U-78875 and Ro 15–1788 dose-dependently blocked the potentiating effect of diazepam. The data represent the mean ± standard deviations from three experiments, each with triplicate measurements.

antagonism of diazepam action by the imidazoquinoxaline. Ro 15–1788, a classical antagonist at the benzodiazepine site, also dose-dependently inhibited the potentiating action of diazepam, reaching 50% block at a concentration of 7 μM.

We have also examined the effect of U-78875 on equilibrium binding of [^{35}S]TBPS to GABA$_A$ receptors (in the presence of 2 μM GABA), because the effect of benzodiazepine ligands on [^{35}S]TBPS binding has been shown to be highly correlated with their ability to potentiate GABA-mediated $^{36}Cl^-$ uptake (32), i.e., benzodiazepine agonists reduced and inverse agonists increased TBPS binding (38, 39). Diazepam at 1 μM inhibited [^{35}S]TBPS binding by 20 ± 3% in the presence of 2 μM GABA. Its action was largely blocked by U-78875 at 1 μM (data not shown). U-78875 by itself, however, marginally affected GABA-induced [^{35}S]TBPS binding, with its inhibition amounting to only 6 ± 4% of the action of diazepam (Table 1). These results are consistent with U-78875 being an antagonist at the benzodiazepine site.

The functional and binding properties in brain membranes of the U-78875 analogs, which vary in the substituents R_1 and R_2, are compared in Table 1. As noted above, the R_1 substituent includes methyl, ethyl, isopropyl, and *t*-butyl, whereas the R_2 group varies from H to Cl, CF_3, and OCH_3. Binding affinity of the analogs was evaluated with measurements of the K_i values for [^{3}H]flunitrazepam binding. Generally, the substituents at R_1 and R_2 produced only marginal changes in the affinity of the drugs for the benzodiazepine site, i.e., only 2–5-fold variations in their K_i values, compared with that of U-78875. Among the substituents, the isopropyl moiety at R_1 induced a binding affinity higher than that with the methyl, ethyl, or *t*-butyl group, whereas the trifluoromethyl group at R_2 seemed to enhance the binding affinity more effectively than the other substituents. Overall, the relatively minor effects of the various substituents on the affinity of the drugs for the benzodiazepine site suggest that these drugs share the same sets of binding pockets in the benzodiazepine-binding region.

Similar binding studies with the cloned receptors, the $\alpha_1\beta_2\gamma_2$ (type I) and $\alpha_3\beta_2\gamma_2$ (type II) subtypes (Table 3), supported the general trend. For instance, the K_i values for the imidazoquinoxalines with $\alpha_1\beta_2\gamma_2$ were very similar to those in brain membranes and were only 2 to 3 times lower than those with the $\alpha_3\beta_2\gamma_2$ subtype. This indicates no marked selectivity of the imidazoquinoxalines for the subtypes of $GABA_A$ receptors.

In contrast, the efficacy of the drugs, as measured by their action on GABA-mediated $^{36}Cl^-$ uptake in the synaptoneurosomes, was markedly affected by the various substituents. The antagonistic activity of U-78875 was converted to an agonistic action by changing R_1 from isopropyl to *t*-butyl (U-82249) or by changing R_2 from H to Cl (U-79098) or OCH_3 (U-89320). The compounds containing methyl or ethyl at R_1 (U-78263, U-78280, and U-81139) showed no noticeable effects on the GABA-mediated $^{36}Cl^-$ uptake. The effects of these drugs, after normalization with respect to that of diazepam, appear to be highly correlated with the relative change in [^{35}S]TBPS binding induced by the drugs, in agreement with earlier studies (32).

Electrophysiology in cloned $GABA_A$ receptors. Measurements of GABA-mediated Cl^- currents were made with the whole-cell configuration of the patch clamp technique. Fig. 4 shows the traces for inward currents, representing the outward movement of Cl^- ions, upon application of 5 μM GABA for 10 sec, with or without the indicated drug (5 μM), in A293 cells expressing $\alpha_1\beta_2\gamma_2$ subunits. A noticeable potentiation of GABA-induced Cl^- current was observed with U-89320, U-82249, and U-79098, in the order of increasing stimulation. U-78263, U-78280, U-78875, and U-81139, on the other hand, produced no noticeable potentiation of the Cl^- current. Table 3 also lists the extent of their potentiation, after normalization to diazepam (5 μM) potentiation or to the GABA (5 μM) response. The results from the cloned cells were very similar to the efficacy measurements in isolated brain membranes. Of particular note is that only U-79098 was more efficacious in the cloned receptors (254 ± 42%) than in brain membranes (103 ± 37%).

Fig. 5 shows similar efficacy studies with the $\alpha_3\beta_2\gamma_2$ subtype. Table 3 compares the relative efficacy values for the imidazoquinoxalines, after normalization to that of diazepam or to the GABA (5 μM) current. One marked difference we observed was that the degree of potentiation by diazepam was much greater in the $\alpha_3\beta_2\gamma_2$ (334 ± 54%) than in the $\alpha_1\beta_2\gamma_2$ subtype (95 ± 20%), as normalized to the amplitude of the current observed with 5 μM GABA. Similarly, the extent of potentiation by the imidazoquinoxaline agonists was also increased in $\alpha_3\beta_2\gamma_2$, by about 2 times, compared with that in $\alpha_1\beta_2\gamma_2$. One exception was U-79098, which displayed more or less the same level of potentiation in the two subtypes, as normalized to the GABA response. Overall, the rank order of efficacy for the imidazoquinoxalines was not changed in the two subtypes.

Fig. 6 shows the competitive nature of interactions between the classical benzodiazepines and the imidazoquinoxaline analogs in the $\alpha_1\beta_2\gamma_2$ subtype. The potentiation of GABA-induced current by diazepam was largely eliminated by U-78875, and that by U-79098 was removed by addition of excess Ro 15–1788, a classical antagonist at benzodiazepine receptors. Similar results were obtained with the $\alpha_3\beta_2\gamma_2$ subtype (data not shown).

Discussion

Using two *in vitro* functional assays in rat cerebrocortical membranes, $^{36}Cl^-$ uptake and [^{35}S]TBPS binding, we have established in this study that U-78875, an imidazoquinoxaline derivative, is an antagonist of high affinity at the benzodiazepine site and that minor variations in its ring substituents produced drugs of agonistic activity. These efficacy measurements in brain cortical membranes were fairly comparable to the electrophysiological measurements in A293 cells expressing type I and type II $GABA_A$ receptors ($\alpha_1\beta_2\gamma_2$ and $\alpha_3\beta_2\gamma_2$, respectively) (20). For instance, the substituent dependency and the efficacy rank order of the imidazoquinoxalines in brain membranes were the same as those for the cloned subtypes of $GABA_A$ receptors (see Tables 1 and 3). This is consistent with the facts that type I and type II represent the major benzodiazepine receptors in the rat cerebrocortex (40) and that the imidazoquinoxalines, like diazepam, are largely nonselective toward the subtypes. Also, the binding properties of the imidazoquinoxalines (K_i values against [^{3}H]flunitrazepam binding) showed no marked subtype selectivity, with only 2–3 times lower K_i values with type I than type II (see Table 3). In an earlier study, diazepam was shown to potentiate GABA-mediated Cl^- currents to a greater extent in the $\alpha_3\beta_2\gamma_2$ than the $\alpha_1\beta_2\gamma_2$ subtype (41). We have also observed in this study that diazepam increased the GABA (5 μM) response by 334 ± 54% in $\alpha_3\beta_2\gamma_2$, compared with 95 ± 20% in the $\alpha_1\beta_2\gamma_2$ subtype. A similar trend was observed with the imidazoquinoxalines, although their level of potentiation in $\alpha_3\beta_2\gamma_2$ was only about 2

TABLE 3

Characterization of binding and functional properties of U-78875 analogs at type I and type II subtypes of $GABA_A$ receptors

The affinity of the U-78875 analogs for the benzodiazepine site was estimated from their ability to inhibit [^{3}H]flunitrazepam (6 nM) binding to membranes prepared from baculovirus-infected SF-9 cells expressing $\alpha_1\beta_2\gamma_2$ (type I) or $\alpha_3\beta_2\gamma_2$ (type II) subunits of $GABA_A$ receptors. The efficacy of the drugs was measured as their ability to potentiate GABA (5 μM)-mediated Cl^- currents in A293 cells expressing $\alpha_1\beta_2\gamma_2$ or $\alpha_3\beta_2\gamma_2$ subunits, using the whole-cell configuration of the patch-clamp technique. The degree of potentiation was normalized to that of diazepam or to the GABA (5 μM) response, as shown in parentheses. The extent of potentiation by diazepam (5 μM) was 95 ± 20%, as normalized to the GABA (5 μM) response, in type I ($\alpha_1\beta_2\gamma_2$) and 334 ± 54% in type II ($\alpha_3\beta_2\gamma_2$). The data represent the mean ± standard error of 4 to 10 separate measurements.

Compound	R_1	R_2	Binding K_i		Cl^- current	
			$\alpha_1\beta_2\gamma_2$	$\alpha_3\beta_2\gamma_2$	$\alpha_1\beta_2\gamma_2$	$\alpha_3\beta_2\gamma_2$
			nM		% of diazepam (% of GABA)	
U-78263	Methyl	H	6.7 ± 0.2	20.8 ± 0.3	8 ± 5 (8)	9 ± 4 (30)
U-78280	Ethyl	H	3.2 ± 0.1	8.2 ± 0.3	16 ± 7 (15)	7 ± 3 (23)
U-78875	Isopropyl	H	1.7 ± 0.2	4.2 ± 0.6	18 ± 10 (17)	9 ± 7 (30)
U-82249	*t*-Butyl	H	3.4 ± 0.5	7.5 ± 0.4	135 ± 25 (128)	68 ± 18 (227)
U-79098	Isopropyl	Cl	0.6 ± 0.1	1.4 ± 0.2	254 ± 42 (241)	81 ± 16 (270)
U-81139	Methyl	CF_3	0.2 ± 0.1	0.4 ± 0.1	0 ± 5 (0)	9 ± 2 (30)
U-89320	Isopropyl	OCH_3	6.1 ± 1.4	20 ± 3.1	82 ± 15 (75)	54 ± 11 (180)

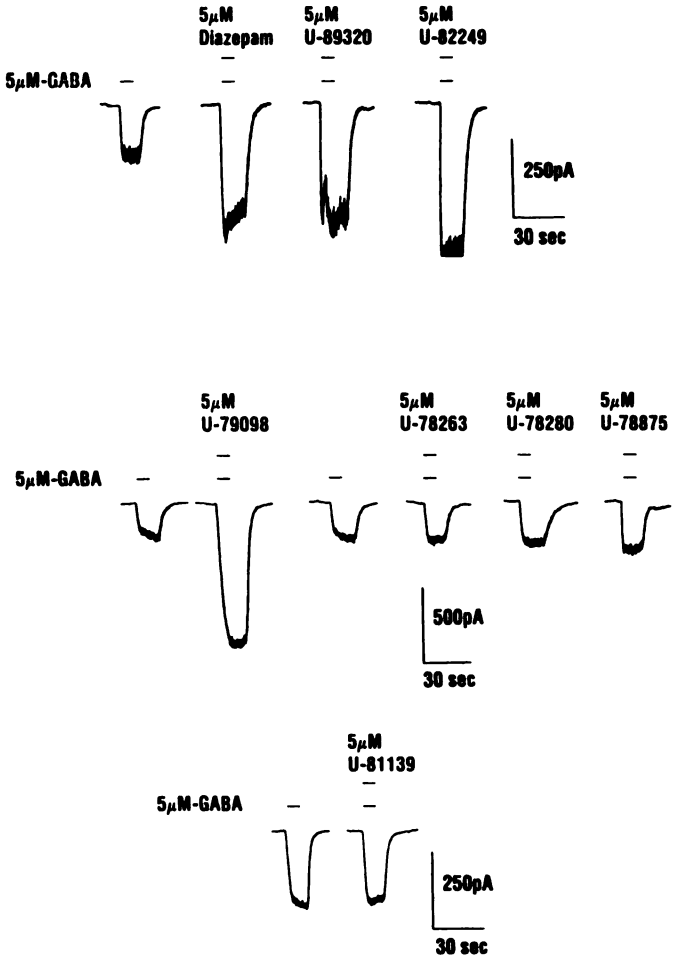

Fig. 4. Effects of U-78875 and its analogs on GABA-mediated Cl^- currents in A293 cells expressing the subtype of GABA$_A$ receptor consisting of $\alpha_1\beta_2\gamma_2$. The Cl^- currents were measured in the whole-cell configuration of the patch-clamp technique, at a holding potential of −60 mV and in the presence of a symmetric Cl^- gradient across the membrane. GABA alone or in combination with a test drug was applied for 10 sec (*bars*). The currents induced by GABA at 5 μM were potentiated by up to 95 ± 20% (n = 10) by diazepam at 5 μM in the cell line. The effects of diazepam and the imidazoquinoxaline analogs were readily washed out. Marked potentiation of the currents was seen with U-89320, diazepam, U-82249, and U-79098, in the order of increasing stimulation, whereas U-78263, U-78280, U-78875, and U-81149 produced no pronounced effect on the currents. The extent of stimulation by the drugs was normalized to that produced by diazepam and is shown in Table 3.

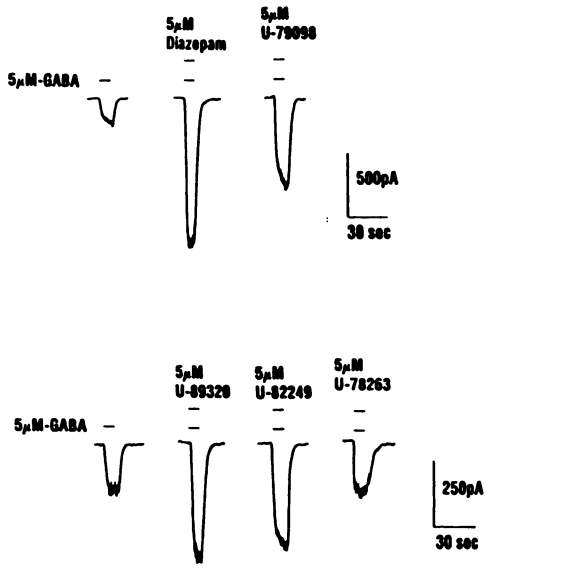

Fig. 5. Representative traces showing the degree of potentiation by the imidazoquinoxalines of GABA-mediated Cl^- currents in A293 cells expressing the subtype of GABA$_A$ receptor consisting of $\alpha_3\beta_2\gamma_2$. The Cl^- currents were measured in the whole-cell configuration of the patch-clamp technique, under the same conditions as described in the legend to Fig. 4. Note that diazepam potentiated GABA-mediated Cl^- currents about three times more in the type II than the type I subtype. The extent of potentiation by the imidazoquinoxalines is compared in Table 3.

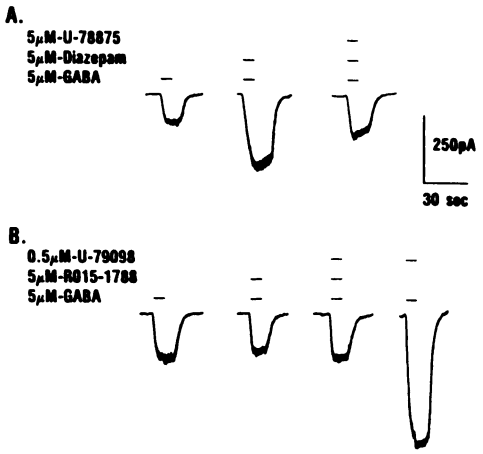

Fig. 6. Competitive interactions between diazepam, a classical benzodiazepine, and the imidazoquinoxaline analogs. The potentiation of GABA-induced Cl^- currents by diazepam at 5 μM was largely eliminated by U-78875 at 5 μM. Also that produced by U-79098 at 0.5 μM was removed by adding Ro 15–1788 to a final concentration of 5 μM.

times greater than that in the $\alpha_1\beta_2\gamma_2$ subtype (see Table 2). One aberration we observed, however, was that the level of potentiation by U-79098 was more or less the same in the two subtypes, as normalized to GABA currents. Accordingly, U-79098 appears to have a greater efficacy (as normalized to that of diazepam) in type I than in type II receptors. This explains, at least in part, the apparent lower efficacy of U-79098 in brain membranes of the mixed subtype populations, compared with that in the $\alpha_1\beta_2\gamma_2$ subtype, especially after normalization to that of diazepam.

In this study, two representative substituents on the agonistic analogs, t-butyl at R_1 (U-82249) and chloro at R_2 (U-79098), produced no significant changes in the MEP at all heteroatoms possibly involved in hydrogen bonding interactions with the benzodiazepine/GABA$_A$ receptor. These results suggest that U-78875 and its analogs share the same binding pockets in the benzodiazepine region, including hydrogen bonding sites proposed as one of the major determinants of GABA$_A$ receptor affinity (6, 9, 11, 12, 14, 15). Three-dimensional computational models obtained from both molecular and quantum mechanical calculations of the analogs indicate that these substituents cause deformations in the substituted portion of the imidazoquinoxaline ring, as measured by changes in the dihedral angle θ. A t-butyl group at R_1 (U-82249) induced an angle of 32°–38°, compared with 16° for U-78875, in which R_1 is isopropyl. When R_2 is chloro (U-79098), θ increased to 43–51°. The fact that the ring system of U-79098 (Cl substituent) could be superimposed on U-78875 except for N_5 and C_6 further indicates that the ring deformation primarily alters the relative spatial positions of R_1 and R_2 groups, without noticeably distorting the rest of the

molecular structure. The puckering of the bottom portion of the imidazoquinoxaline ring appears to be necessary but not sufficient for agonistic activity of the analogs. For example, U-81139, containing the highly puckered ring system with a dihedral angle θ of 49.8°, showed no agonistic activity. One conspicuous structural difference is that U-81139 has a methyl group at R_1, whereas agonistic analogs contain larger, more lipophilic groups, such as isopropyl or *t*-butyl. From these observations, we propose that the ring deformation induces an interaction between the bulky alkyl group (R_1) and a hydrophobic pocket on the receptor, which in turn plays a key role in inducing the conformational changes in the receptor necessary for agonistic activity. One interesting point to emphasize is that the bulky alkyl group appears to be out-of-plane, relative to the imidazoquinoxaline ring.

In generalizing our observations to other ligands, it is useful to identify the two key heteroatoms in the imidazoquinoxaline series that might correspond to those proton acceptors proposed to be necessary for receptor recognition for other ligands (6, 9, 11, 12, 14). The selection is not straightforward, due to the presence of five candidates, $O_{1'}$, O_{14}, N_2, $N_{2'}$, and $N_{4'}$, in each of the imidazoquinoxaline analogs. However, one possibility is suggested by the locations of calculated MEP minima on van der Waals surfaces of the low energy conformations of U-78875, U-79098, and U-82249. In each case, the two points of lowest MEP occur near N_2 and midway between O_{14} and $N_{4'}$. Furthermore, the MEP minimum near N_2 may be assigned to correspond to σ_1 in the model of Tebib *et al.* (14), which is equivalent to site 2 and H_1 in the models of Villar *et al.* (12) and Hollinshead *et al.* (11), respectively. This assignment puts the bulky alkyl substituent at N_5 in the general region occupied by the 5-phenyl substituent of diazepam in nearly all the models for agonists. With these arrangements, our findings provide direct support for the proposals of several groups (9, 11, 12, 14). For instance, Tebib *et al.* (14) have proposed the existence of an OPR in the benzodiazepine receptor, which can accommodate the 5-phenyl group of diazepam or similar bulky groups of other ligands and is responsible for the agonistic property. This proposed OPR is analogous to the hydrophobic pocket we have assigned to interact with the bulky alkyl group at N_5 of the imidazoquinoxaline ring system. The functional importance of this region was also noted as the lipophilic pocket by Hollinshead *et al.* (11), as out-of-plane displacement zone 2 by Borea *et al.* (9), and as the agonistic site by Villar *et al.* (12). Our results further show that the agonistic activity seems to be proportional to the degree of ring puckering among the analogs containing the isopropyl group at N_5 (Tables 1 and 3). This further underscores the extent of interaction with OPR as one of the key determinants for agonistic activity.

Although the OPR appears to be common in classical benzodiazepines and various nonbenzodiazepine ligands, it may not be the only region responsible for the functional consequences. In some models for nonagonists, a proton donor site has been proposed as one of the requirements for antagonists, by Codding and Muir (6) and Hollinshead *et al.* (11). The distance between the center of an aromatic ring and a proton-accepting atom has been proposed to be >6 Å for nonagonists and <6 Å for agonists (8). Also, the relative angle between the two anchoring hydrogen bond acceptor sites and the most lipophilic aromatic region has been pointed out, by Villar *et al.* (12), to change from about 145° for agonists to 95–115° for antagonists. One of the possible sources for these discrepancies may be the existence of more than one binding pocket responsible for the functionality of the benzodiazepine receptors. In this study, we have shown that the rigid and largely planar ring structure of the imidazoquinoxaline derivatives is useful in assigning unambiguously the OPR as a key group for agonistic activity. We believe that other modifications of the imidazoquinoxaline ring could lead to the discovery of other groups of functional significance, with the reliable *in vitro* functional assays used in this study.

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