Differential affinity of dihydroimidazoquinoxalines and diimidazoquinazolines to the $\alpha 1\beta 2\gamma 2$ and $\alpha 6\beta 2\gamma 2$ subtypes of cloned GABA_A receptors

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1 In this study, we compared two series of newly discovered ligands for their selectivity to benzodiazepine sites in the $\alpha 1\beta 2\gamma 2$ and the $\alpha 6\beta 2\gamma 2$ subtypes of cloned γ -aminobutyric acid_A (GABA_A) receptors, the latter being unique in not interacting with classical benzodiazepines.

2 The prototype compounds, U-85575 (12-chloro-5-(5-cyclopropyl-1',2',4'-oxadiazol-3'-yl)-2,3-dihydro-diimidazo[1,5-a;1,2-c]quinazoline), and U-92330 (5-acetyl-3-(5'-cyclopropyl-1',2',4'-oxadiazole-3'-yl)-7-chloro-4,5-dihydro[1,5-a]quinoxaline), appear to share an overlapping recognition site with classical benzodiazepines on the GABA_A receptor, because their potentiation of GABA-mediated Cl⁻ currents in both subtypes were sensitive to Ro 15-1788, a classical benzodiazepine antagonist.

3 Minor changes in the ring substituents of the drugs reduced their affinity to the $\alpha 6\beta 2\gamma 2$ subtype more pronouncedly than to the $\alpha 1\beta 2\gamma 2$ subtype. The diimidazoquinazoline containing a 2-methyl group which projected below the plane of the rigid ring showed a markedly lower affinity to the $\alpha 6\beta 2\gamma 2$ subtype as compared to its stereoisomer having the methyl group above the plane of the ring. Also, the dihydroimidazoquinoxalines containing the 5-benzoyl group showed a lower affinity to the $\alpha 6\beta 2\gamma 2$ subtype than the 5-acetyl counterpart. In particular, the 5-benzoyl analogue containing a 6-fluoro group showed no interaction with the $\alpha 6\beta 2\gamma 2$ subtype even at the concentration of 10 μ M, probably due to stabilization of the benzoyl group in the out-of-plane region by the steric and electrostatic effects of the 6-fluoro group.

4 We propose that the benzodiazepine site of the $\alpha 6\beta 2\gamma 2$ subtype shares overlapping regions with that of the $\alpha 1\beta 2\gamma 2$ subtype, but has a sterically restricted out-of-plane region, which may be also incompatible with the 5-phenyl group of classical benzodiazepines.

Keywords: GABA_A receptor; $\alpha 1\beta 2\gamma 2$; $\alpha 6\beta 2\gamma 2$; diimidazoquinazoline; dihydroimidazoquinoxaline; benzodiazepine site

Introduction

Benzodiazepines and related non-benzodiazepine hypnotic or anxiolytic agents interact with γ -aminobutyric acid (GABA_A) receptors, the supermolecular receptor-Cl⁻ channel complexes made of heteromultimeric subunits (Costa, 1979; Yokohama *et al.*, 1982; Haefely *et al.*, 1985; Barnard *et al.*, 1987; Schofield, 1989; Sieghart, 1992). In mammalian brains, several subunits with families of isotypes exist for GABA_A receptors (Schofield, 1989; Sigel *et al.*, 1990; Verdoorn *et al.*, 1990; Sieghart, 1992). The most widespread subunits in the brain, $\alpha 1$, $\beta 2$, and $\gamma 2$, have been expressed in human kidney cells, and the cloned receptors ($\alpha 1\beta 2\gamma 2$) have been shown to interact with ligands for benzodiazepine sites (Pritchett *et al.*, 1989a,b; Puia *et al.*, 1991). Interestingly, different isotypes of the α subunit, when expressed with the $\beta 2$ and $\gamma 2$ subunits, influenced the selectivity of the cloned receptors to benzodiazepine ligands: The $\alpha 1\beta 2\gamma 2$ subtype showed higher affinity to zolpidem (imidazopyridines), Ro 15-1788 and the β -carboline than the $\alpha 2\beta 2\gamma 2$ and $\alpha 3\beta 2\gamma 2$ subtypes (Pritchett *et al.*, 1989a,b; Puia *et al.*, 1992). The three subtypes ($\alpha 1\beta 2\gamma 2$, $\alpha 2\beta 2\gamma 2$ and $\alpha 3\beta 2\gamma 2$) interact with the classical benzodiazepines with nearly equal affinity (Pritchett & Seeburg, 1990; Puia *et al.*, 1992). The $\alpha 6\beta 2\gamma 2$ subtype in cerebellar granule cells differs from the above ones in that it does not accommodate the classical benzodiazepines, although it shares several other ligands for benzodiazepine sites with the other subtypes (i.e., Ro 15-4513; Luddens *et al.*, 1990). In the course of studying subtype selectivity, we discovered two new series of ligands which displayed markedly differential affinity to the $\alpha 1\beta 2\gamma 2$ and $\alpha 6\beta 2\gamma 2$ subtypes, depending on their particular substituents.

Here we report that U-92330, 5-acetyl-3-(5'-cyclopropyl-1',2',4'-oxadiazole-3'-yl)-7-chloro-4,5-dihydroimidazo[1,5-a]quinoxaline, and U-85575, 12-chloro-5-(5-cyclopropyl-1',2',4'-oxadiazol-3'-yl)-2,3-dihydro-diimidazo[1,5-a;1,2-c]quinazoline (Figure 1), interact with both $\alpha 1\beta 2\gamma 2$ and $\alpha 6\beta 2\gamma 2$ subtypes, but the 5-benzoyl analogues of U-92330 and the 2-methyl analogues of U-85575 display markedly reduced affinities to $\alpha 6\beta 2\gamma 2$ as compared with their affinity to the $\alpha 1\beta 2\gamma 2$ subtype. It appears that the presence of an alkyl or aromatic group in the out-of-plane region with respect to the ring system reduces their affinity to the $\alpha 6\beta 2\gamma 2$ subtype due to the presence of at least one sterically restricted region as compared to that of the $\alpha 1\beta 2\gamma 2$ subtype.

Methods

GABA_A receptor expression

DNA manipulations and general baculovirus methods (Sf9 cell cultivation, infection and isolation, and purification of recombinant viruses) were performed as described elsewhere (Carter *et al.*, 1992). The Sf-9 cells were infected at a multiplicity of infection of three plaque-forming units of viruses; AcNPV- $\alpha 1$ or - $\alpha 6$, AcNPV- $\beta 2$ and AcNPV- $\gamma 2$. Infected cells were used for electrophysiological measurements at 48 h post infection, or for membrane preparations at 60 h post infection.

The stable cell-lines expressing $\alpha 1$ or $\alpha 6$ (Khrestchatsky *et al.*, 1989; Luddens *et al.*, 1990), $\beta 2$ (Ymer *et al.*, 1989), and $\gamma 2$ (Shivers *et al.*, 1989) subunits of GABA_A receptors were derived by transfection of plasmids containing cDNA and a plasmid encoding G418 resistance into the human kidney

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Figure 1 Chemical structures of the diimidazoquinazolines (a) and dihydroimidazoquinoxalines (b).

cells (A293 cells) as described elsewhere (Hamilton *et al.*, 1993). After two weeks of selection in 1 mg ml^{-1} G418, cells positive for all three GABA_A receptor mRNAs by Northern blotting were used for electrophysiology to measure GABA-induced Cl^- currents.

Equilibrium binding

For the binding study, Sf-9 cells infected with baculovirus carrying cDNAs for $\alpha 1$ or $\alpha 6$, $\beta 2$ and $\gamma 2$ subunits were harvested in 21 batches 60 h post infection. The membranes were prepared following the procedures described elsewhere (Carter *et al.*, 1992), and were stored at -80°C in a solution containing 300 mM sucrose, 5 mM Tris/HCl, pH 7.5, and glycerol to a final concentration of 20%. Equilibrium binding of [^3H]-flunitrazepam or [^3H]-Ro 15-4513 (ethyl 8-azido-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5-a][1,4]benzodiazepine-3-carboxylate) to the GABA_A receptors was measured in a 500 μl volume of normal saline containing 6 nM [^3H]-flunitrazepam or [^3H]-Ro 15-4513, varying concentrations of test ligands, and 50 μg of membrane protein. The mixture was incubated for 60 min at 4°C , filtered over Whatman glass fibre filters, and washed four times with cold normal saline. The filter was then counted for radioactivity in the presence of a scintillation cocktail (Insta gel).

Electrophysiology

The whole cell configuration of the patch clamp techniques (Hamill *et al.*, 1981) was used to record to GABA-mediated Cl^- currents as described previously (Verdoorn *et al.*, 1990). The pipette solution contained (mM) CsCl 140, EGTA 11, MgCl_2 2, ATP 0.5 and HEPES 10, pH 7.3, and the external solution contained (mM): NaCl 130, KCl 5, MgCl_2 1, CaCl_2 1.8 and HEPES 5, pH 7.2, (normal saline). GABA and drugs were dissolved at the indicated concentrations in the external solution, and were applied through a Y-tube (Murase *et al.*, 1989) placed within 100 μm of the cells. The current through the pipette was recorded with an Axopatch 1D amplifier and a CV-4 headstage (Axon Instrument Co.). The currents were recorded with Gould Recorder 220. Experiments were carried out at a holding potential of -60 mV at room temperature ($21\text{--}24^\circ\text{C}$).

Results and discussion

Functional characteristics

U-92330 was examined for its functional characteristics in the $\alpha 1\beta 2\gamma 2$ and $\alpha 6\beta 2\gamma 2$ subtypes expressed in Sf-9 cells (Figure 2a). The drug at $1 \mu\text{M}$ (saturating concentration) enhanced GABA-mediated Cl^- currents by $112 \pm 25\%$ ($n = 4$) in $\alpha 1\beta 2\gamma 2$ and by $148 \pm 43\%$ ($n = 3$) in $\alpha 6\beta 2\gamma 2$, as normalized to GABA ($5 \mu\text{M}$) response. Its potentiation of Cl^- current in the two subtypes was overcome by Ro 15-1788 (ethyl-8-fluoro-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5-a][1,4]benzodiazepine-3-carboxylate; $10 \mu\text{M}$), suggesting that the drug shares an overlapping recognition site with the classical benzodiazepine antagonist. Interestingly, Ro 15-1788 enhanced GABA mediated Cl^- currents by $76 \pm 40\%$ ($n = 3$) in the $\alpha 6\beta 2\gamma 2$, but not in the $\alpha 1\beta 2\gamma 2$ subtype. We obtained similar results with the cloned receptors expressed in human embryonic kidney cells (A293 cells) (Figure 2b). In A293 cells, U-92330 at $1 \mu\text{M}$ enhanced Cl^- currents by $140 \pm 27\%$ ($n = 5$) in the $\alpha 1\beta 2\gamma 2$ and by $257 \pm 35\%$ ($n = 4$) in the $\alpha 6\beta 2\gamma 2$ subtype in a Ro 15-1788-sensitive way. Also, Ro 15-1788 increased Cl^- currents by $96 \pm 38\%$ ($n = 6$) only in the $\alpha 6\beta 2\gamma 2$ subtype. U-92038, the 5-benzoyl counterpart of U-92330, was also an agonist in the two subtypes expressed in A293 cells; the drug at a concentration of $5 \mu\text{M}$ increased GABA-mediated Cl^- currents by $155 \pm 15\%$ ($n = 3$) in the $\alpha 1\beta 2\gamma 2$ and by $203 \pm 10\%$ ($n = 3$) in the $\alpha 6\beta 2\gamma 2$ subtype.

U-85575, representing the diimidazoquinazolines, was also an agonist in the subtypes expressed in A293 cells; the drug at $5 \mu\text{M}$ enhanced Cl^- current by $45 \pm 10\%$ in the $\alpha 1\beta 2\gamma 2$ subtype in a Ro 15-1788-sensitive manner (data not shown), as found earlier (Im *et al.*, 1992) and by $126 \pm 20\%$ in the $\alpha 6\beta 2\gamma 2$ subtype.

It has been proposed in an earlier study (Kleingoor *et al.*, 1991) that inverse agonists, but not full agonists, modulate the $\alpha 6\beta 2\gamma 2$ subtype in A293 cells. Our current finding that the dihydroimidazoquinoxalines, the diimidazoquinazolines and Ro 15-1788 were agonists in the $\alpha 6\beta 2\gamma 2$ indicates that the chemical moiety responsible for agonistic activity for the $\alpha 6\beta 2\gamma 2$ is different from those for the $\alpha 1\beta 2\gamma 2$ subtype. Furthermore, the benzodiazepine site in $\alpha 6\beta 2\gamma 2$ is involved in a positive as well as negative modulation of the receptor function.

Binding characteristics

In agreement with earlier studies, the classical benzodiazepines like flunitrazepam and diazepam had no effect on binding of [^3H]-Ro 15-4513 in the cell membranes expressing the $\alpha 6\beta 2\gamma 2$ subtype of GABA_A receptors (expressed in Sf-9 cells using baculovirus) even at concentrations of $5 \mu\text{M}$ (data not shown). In Table 1, we compare the K_i values of U-85575 and its 2-methyl stereoisomers in cell membranes expressing the $\alpha 1\beta 2\gamma 2$ and $\alpha 6\beta 2\gamma 2$ subtypes. U-85575 blocked binding of [^3H]-flunitrazepam with a K_i value of 0.3 nM in the $\alpha 1\beta 2\gamma 2$ subtype as found earlier, and that of [^3H]-Ro 15-4513 with a K_i value of 68 nM in the $\alpha 6\beta 2\gamma 2$ subtype. The similar ratio of the K_i value in $\alpha 1\beta 2\gamma 2$ to that in $\alpha 6\beta 2\gamma 2$ (about 200) was also observed with U-90168, the analogue of U-85575 with the 2-methyl substituent projected above the plane-of-the-ring, its K_i being 1 nM in $\alpha 1\beta 2\gamma 2$ and 181 nM in $\alpha 6\beta 2\gamma 2$. The ratio, however, became greater (860) with U-90167, a stereoisomer of U-90168, with its methyl substituent projected below the plane-of-the-ring; its K_i value was 3.8 nM in $\alpha 1\beta 2\gamma 2$ and 3270 nM in $\alpha 6\beta 2\gamma 2$. This difference between the stereoisomers indicates that the benzodiazepine site on the $\alpha 6\beta 2\gamma 2$ subtype (as compared to $\alpha 1\beta 2\gamma 2$) has a sterically restricted region where the 2-methyl group of U-90167 (projected below the plane-of-the-ring) comes in contact.

The prototype compound of the dihydroimidazoquinoxalines, U-92330, blocked binding of [^3H]-flunitrazepam in the $\alpha 1\beta 2\gamma 2$ subtype with a K_i value of $2.4 \pm 0.1 \text{ nM}$ and blocked

a Sf-9 cells infected with baculo virus **b Human embryonic kidney cells (A293 Cells)**

Figure 2 Traces showing the ability of U-92330 to potentiate γ -aminobutyric acid (GABA)-induced Cl^- currents and its sensitivity to Ro 15-1788 in the $\alpha 1\beta 2\gamma 2$ and the $\alpha 6\beta 2\gamma 2$ subtypes of GABA_A receptors expressed in (a) Sf-9 insect cells or (b) human embryonic kidney cells. Whole cell currents induced by GABA at 5 μM were measured in the absence or presence of U-92330 (5 μM), Ro 15-1788 (10 μM), or a combination of the two. The currents were induced under a holding potential of -60 mV and a symmetrical Cl gradient.

binding of [^3H]-Ro 15-4513 in the $\alpha 6\beta 2\gamma 2$ subtype with a K_i value of 35 ± 8.3 nM (Table 1). We compared the affinity of three pairs of U-92330 analogues containing either the 5-acetyl or 5-benzoyl moiety in the two subtypes. In $\alpha 1\beta 2\gamma 2$, the K_i values were very similar among the three pairs, ranging from 0.4 to 1.7 nM, although the K_i values of the 5-acetyl analogues tended to be slightly higher than the 5-benzoyl analogues (Table 1). In $\alpha 6\beta 2\gamma 2$, however, the 5-acetyl analogues displayed considerably lower K_i values (higher affinity) as compared to the 5-benzoyl analogues. U-92330, U-92593 and U-92842 (the 5-acetyl analogues) blocked the binding of [^3H]-Ro 15-4513 in cell membranes expressing the $\alpha 6\beta 2\gamma 2$ subtype with the K_i value of 35 ± 8.3 , 107 ± 21 and 81 ± 1.1 nM, respectively, while the corresponding 5-benzoyl analogues, U-92038, U-92594 and U-92130 displayed a K_i value of 1024 ± 49 , $> 10,000$ and 681 ± 4.2 nM, respectively. In particular, U-92594, the 5-benzoyl analogue containing a fluoro substituent at C6 (R2), had no interaction with the $\alpha 6\beta 2\gamma 2$ subtype, even at a concentration of 10,000 nM.

The overlay of the two common rings, the benzene and imidazole moieties, of the diimidazoquinazolines and the dihydroimidazoquinoxalines shows that the 5-benzoyl group of U-92594 may occupy the same general region as the 2-methyl group of U-90167 (see Figure 1). Furthermore, molecular mechanics studies using the AMBER force field show that the 5-benzoyl group of U-92594 is out-of-the-plane with respect to the ring system, as is the 2-methyl group of U-90167 (Figure 3). The exact location of the benzoyl group of U-92594, however, is not as clear as the 2-methyl group of

Table 1 Comparison of binding affinity of the diimidazoquinazoline and the dihydroimidazoquinoxaline analogues in cloned γ -aminobutyric acid_A (GABA_A) receptor subtypes

Diimidazoquinazolines	Binding, K_i (nM)				
	R_1	R_2	$\alpha 1\beta 2\gamma 2$	$\alpha 6\beta 2\gamma 2$	
U-85575	H	H	0.3 ± 0.1	68 ± 5	
U-90167	CH_3	H	3.8 ± 0.5	3270 ± 250	
U-90168	H	CH_3	1.0 ± 0.2	181 ± 42	

Dihydroimidazoquinoxaline	Binding, K_i (nM)				
	R_1	R_2	R_3	$\alpha 1\beta 2\gamma 2$	$\alpha 6\beta 2\gamma 2$
U-92330	Cl	H	Acetyl	1.7 ± 0.4	35 ± 8
U-92038	Cl	H	Benzoyl	1.3 ± 0.1	1024 ± 49
U-92593	H	F	Acetyl	0.8 ± 0.2	107 ± 21
U-92594	H	F	Benzoyl	0.4 ± 0.1	$> 10,000$
U-91842	F	H	Acetyl	0.6 ± 0.1	81 ± 1
U-92130	F	H	Benzoyl	0.4 ± 0.1	681 ± 4

Binding of [^3H]-flunitrazepam was measured in the membranes from Sf-9 insect cells expressing the $\alpha 1\beta 2\gamma 2$ subtype and that of [^3H]-Ro 15-4513 in the insect cell membranes expressing the $\alpha 6\beta 2\gamma 2$ subtype as described in the Methods. The IC_{50} value was obtained from dose-response curves consisting of six different concentrations, and was converted to K_i s by use of the equation, $K_i = \text{IC}_{50} / (1 + [\text{Test ligand}] / K_d \text{ of flunitrazepam or Ro 15-4513})$. The data represent the mean $\pm$ standard errors from at least three separate experiments. It should be noted that the K_i values of several drugs were obtained with [^3H]-Ro 15-4513 in the $\alpha 1\beta 2\gamma 2$ subtype and were not noticeably different from those obtained with [^3H]-flunitrazepam.

U-90167

U-92594

Figure 3 Side-view of minimum energy structures of U-90167 and U-92594.

U-90167, due to amide rotamers. Perhaps the steric and electrostatic effects of the adjacent fluorine may restrict the mobility of the benzoyl group and bring it in contact with a sterically restricted region on the $\alpha 6\beta 2\gamma 2$ subtype as with the 2-methyl group of U-90167. The close interaction of the C6 fluoro substituent and the 5-benzoyl group was further underscored by the observation that the 5-benzoyl analogues with the C7-fluoro substituent (U-92130) have a much higher affinity for the $\alpha 6\beta 2\gamma 2$ subtype ($K_i = 681$ nM) than the 6-fluoro-5-benzoyl analogue, U-92594 ($K_i > 10,000$).

Earlier studies (Tebib *et al.*, 1987) have assigned the 5-phenyl group of diazepam to be out-of-plane with respect to the parent ring system. This could account, at least in part,

for the lack of interaction of diazepam with the $\alpha 6\beta 2\gamma 2$ subtype of GABA_A receptors. It appears that the benzodiazepine site of the $\alpha 6\beta 2\gamma 2$ subtype shares many common recognition regions with that of the $\alpha 1\beta 2\gamma 2$ subtype, but has at least a sterically restricted area which poorly accommodates bulky hydrophobic groups in the out-of-plane region.

Wieland *et al.* (1992) have shown in the $\alpha 6\beta 2\gamma 2$ subtype

that mutation of arginine-100 to histidine in the $\alpha 6$ subunit confers the ability to interact with classical benzodiazepines, thus confirming the equivalence of arginine-100 in $\alpha 6$ to histidine-101 in the other α subunits ($\alpha 1$, $\alpha 2$, $\alpha 3$ or $\alpha 5$), which play a key role for interaction with classical benzodiazepines. It is tempting to speculate that the Arg-100 in the $\alpha 6$ subunit may be responsible for creating the sterically restricted area in the benzodiazepine recognition site of the $\alpha 6\beta 2\gamma 2$ subtype.

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