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Potentiation of γ-Aminobutyric Acid-Induced Chloride Currents by Various Benzodiazepine Site Agonists with the α1γ2, β2γ2 and α1β2γ2 Subtypes of Cloned γ-Aminobutyric Acid Type A Receptors

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SUMMARY

Previous studies with cloned γ-aminobutyric acid type A receptors expressed in human embryonic kidney cells have indicated that the α1β2γ2 and α1γ2 (but not α1β2) subtypes have benzodiazepine sites. We found in this study that even the β2γ2 subtype displays γ-aminobutyric acid-induced Cl^- currents that are potentiated by triazolam (a triazolobenzodiazepine). The maximal efficacy of the drug among the subtypes was highest with the α1β2γ2 subtype, followed by the α1γ2 and β2γ2 subtypes. These observations led us to compare the ability of several benzodiazepine site agonists of diverse chemical structures to potentiate Cl^- currents with these subtypes. With the α1γ2 subtype, diazepam, alpidem, zolpidem, CI-218872, zopiclone, U-79098 (an imidazoquinoxaline derivative), and U-90167 (a diimidazoquinazoline derivative) at 5 μM potentiated Cl^- currents to essentially similar levels (slightly lower for a few ligands), compared with those with the α1β2γ2 subtype. With the β2γ2 subtype, the type 1 ligands zolpidem, alpidem, and CI-218872 showed no or very low levels of potentiation, whereas less selective ligands such as diazepam, zopiclone, U-78098, and U-90167 displayed levels of Cl^- current potentiation comparable to those observed with the subtypes containing the α1 and γ2 subunits. These data indicate that, in the presence of γ2, β2 may substitute for α1 in forming the benzodiazepine site of limited sensitivity to the type 1 ligands. It appears that individual ligands for benzodiazepine sites have their own sets of interacting domains, which are distributed in α1 and γ2, and the agonistic activity of type 1 ligands may be more dependent on the α1-specific domains than is that of less selective ligands.

The benzodiazepine site is a well known allosteric modulatory site on $GABA_A$ receptors in the brain that accommodates not only classical benzodiazepines but also nonbenzodiazepine hypnotic agents of various chemical structures (1–5). Recent cloning of $GABA_A$ receptor subunits showed the existence of several families of subunits with various isotypes (6–10). Expression of the most ubiquitous subunits, α1 (or α2), β2 (or β1), and γ2, in human embryonic kidney cells (6–15) produced functional receptors with neuronal characteristics and provided two important findings with respect to the benzodiazepine site, i.e., 1) benzodiazepines interact with the α1β2γ2 but not the α1β2 subtype (14) and 2) the α isotype of αxβ2γ2 subtypes determines ligand selectivity (10, 15). Furthermore, the α subunits were photoaffinity labeled with flunitrazepam (a photosensitive benzodiazepine ligand) (10, 16). Recently it was reported that the kidney cell line expressing the α1 and γ2 subunits (although initially transfected with cDNAs for the α1, β2, and γ2 subunits) expressed $GABA_A$ receptors with benzodiazepine-binding properties similar to those of the α1β2γ2 subtype (17). This supports the notion that the α and γ subunits play important roles in forming a benzodiazepine site.

In the course of characterizing various templates for $GABA_A$ receptor ligands, however, we found that even the β2γ2 subtype displayed benzodiazepine-sensitive GABA-induced Cl^- currents, indicating the versatility of benzodiazepine sites. In this study, we compared various classes of benzodiazepine site agonists for their ability to affect GABA-mediated Cl^- currents with the α1β2γ2, α1γ2, and β2γ2 subtypes. It appears that the β2γ2 subtype interacts with a narrower spectrum of ligands than do the other subtypes. Notable ligands not interacting with the β2γ2 subtype include imidazopyridines and CI-218872 [type 1 (α1)-selective compounds].

Materials and Methods

Stable cell lines expressing the indicated combinations of α1 (18), β2 (19), and γ2 (20), or of any two subunits of $GABA_A$ receptors, were derived by transfection of plasmids containing appropriate cDNAs and a plasmid encoding G418 resistance into human embryonic kidney cells

ABBREVIATIONS: GABA, γ-aminobutyric acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

(A293) (21). After 2 weeks of selection in 1 mg/ml G418, resistant cells were assayed by Northern blotting for the ability to synthesize $GABA_A$ receptor mRNA. Positive cells with appropriate subunit combinations were used for electrophysiology to measure GABA-induced Cl^- currents.

The whole-cell configuration of the patch-clamp technique (22) was used to record GABA-mediated Cl^- currents in A293 cells expressing various combinations of $GABA_A$ receptor subunits, as described earlier (23). 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) 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 the holding potential of −60 mV at room temperature (21–24°).

Results

GABA-induced Cl^- currents were measured using the whole-cell configuration of the patch-clamp technique in A293 cells expressing the indicated combinations of $GABA_A$ receptor subunits. Triazolam, a triazolobenzodiazepine with high affinity for the $GABA_A$ receptor, at 2 μM (a saturating concentration) had no effect on GABA-induced Cl^- currents with the α1β2 subtype (data not shown), in agreement with earlier reports, but potentiated Cl^- currents with the α1β2γ2, α1γ2, and β2γ2 subtypes of $GABA_A$ receptors (Fig. 1). The latter three subtypes of $GABA_A$ receptors, although uniformly responsive to triazolam, could be differentiated by a substituted pyrazinone (U-92813), a novel $GABA_A$ receptor ligand that interacts only with receptors containing the α and β subunits and not with those missing either the α1 or β2 subunit (Fig. 1) (24).

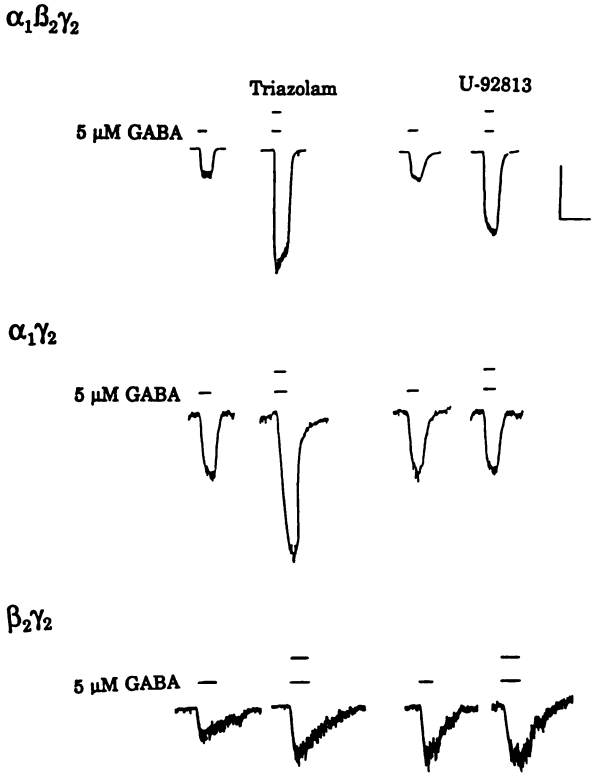

Fig. 1. Effect of triazolam and U-92813 (a substituted pyrazinone) (24) on GABA-induced Cl^- currents with the α1β2γ2, α1γ2, and β2γ2 subtypes of cloned $GABA_A$ receptors. Cl^- currents were measured, using the whole-cell configuration of the patch-clamp technique, in A293 cells expressing the subtypes of $GABA_A$ receptors. The holding potential was −60 mV under a symmetrical Cl^- gradient. Triazolam at 2 μM potentiated GABA (5 μM)-induced Cl^- currents with the three subtypes, whereas U-92813 [1-(furfuryl)-3,5-dichloro-6-phenylpyrazinone] increased Cl^- currents only with the α1β2γ2 subtype. *Scale in the upper right corner, vertical bar*, 500 pA (*upper*) or 200 pA (*middle* and *lower*); *horizontal bar*, 30 sec.

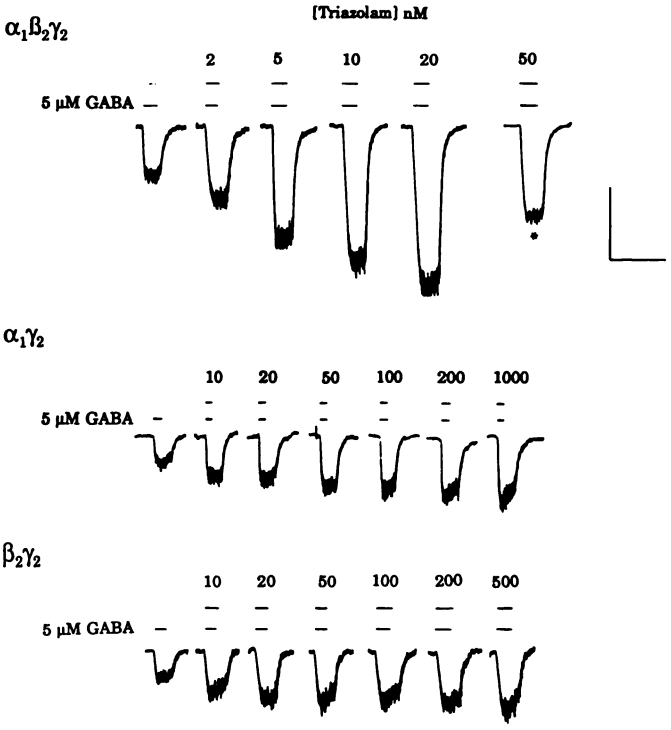

Fig. 2. *Traces* showing dose-dependent potentiation of GABA-induced Cl^- currents by triazolam with the α1β2γ2, α1γ2, and β2γ2 subtypes of $GABA_A$ receptors. Cl^- currents were induced with GABA (5 μM) in the absence or presence of triazolam at the indicated concentrations. *Scale in the upper right corner, vertical bar*, 500 or 1000 pA (*) (*upper*), 500 pA (*middle*), or 200 pA (*lower*); *horizontal bar*, 30 sec.

Figs. 2 and 3 show dose-response profiles for potentiation by triazolam of GABA-induced Cl^- currents with the α1β2γ2, α1γ2, and β2γ2 subtypes. The data were analyzed using the following logistic equation (25): $E = E_m \times [\text{drug}]^n/(EC_{50}{}^n + [\text{drug}]^n)$, 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 EC_{50} is the concentration of drug needed to produce 50% of the maximal effect. From the analysis (Fig. 3), we obtained an EC_{50} of 5 ± 0.7 nM, E_m of 201%, and n of 1.4 ± 0.3 for triazolam with the α1β2γ2 subtype, 18 ± 3 nM, 117 ± 34%, and 0.8 ± 0.2 with the α1γ2 subtype, and 8.5 ± 0.7 nM, 79 ± 3%, and 1.4 ± 0.2 with the β2γ2 subtype, respectively. It is apparent that the subunit variations noticeably influence the maximal level of potentiation for triazolam.

The dose-dependent potentiation of Cl^- currents by triazolam indicates the presence of a benzodiazepine site not only in the α1β2γ2 and α1γ2 subtypes but also in the β2γ2 subtype, and this prompted us to compare the ability of several benzodiazepine site agonists to potentiate Cl^- currents. Fig. 4 shows representative traces of GABA (5 μM)-induced Cl^- currents,

with or without the indicated agonists, with the $\alpha1\gamma2$ and $\beta2\gamma2$ subtypes. Table 1 shows the average levels of potentiation, as normalized to the current observed with GABA at 5 μM, for various known agonists for benzodiazepine sites, at a fixed concentration of 5 μM. With the $\alpha1\beta2\gamma2$ subtype, the potentiation level for agonists ranged from approximately 100 to 250%. U-79098 (imidazoquinoxaline) (26) was the most efficacious (254%), followed by U-90167 (diimidazoquinazoline) (220%) (27), alpidem (imidazopyridines) (201%), and triazolam (a classical benzodiazepine) (196%). Zolpidem (another imidazopyridine), diazepam, Cl-218872 (triazolopyridazine), and zopiclone (cyclopyrrolone) enhanced Cl^- currents by approximately 100%. With the $\alpha1\gamma2$ subtype, most ligands showed potentiation levels similar to those observed with the $\alpha1\beta2\gamma2$ subtype, except for triazolam, U-79098, and U-90167 (Fig. 4A; Table 1). These three drugs displayed about 50–60% of their potentiation levels with the $\alpha1\beta2\gamma2$ subtype, and their efficacy did not improve even at higher drug concentrations (i.e., 10 μM). These quantitative variations with some ligands may reflect the influence of the $\beta2$ subunit on the benzodiazepine site, but overall the benzodiazepine site in the $\alpha1\gamma2$ subtype appears to be similar to that in the $\alpha1\beta2\gamma2$ subtype, as judged by its interactions with diverse substrates including benzodiazepines, triazolobenzodiazepine, imidazopyridines (alpidem), triazolopyridazines (Cl-218872), cyclopyrrolones (zopiclone), imidazoquinoxalines, and diimidazoquinazolines.

With the $\beta2\gamma2$ subtype, only selective agonists potentiated Cl^- currents (Fig. 4B; Table 1). Zopiclone, the imidazoquinoxaline U-79098, and the diimidazoquinazoline U-90167 potentiated the Cl^- currents to levels nearly equal to those observed with the $\alpha1\gamma2$ subtype. Classical benzodiazepines (diazepam and triazolam) potentiated currents up to approximately 50% of their levels with the $\alpha1\gamma2$ subtype. The imidazopyridines (particularly zolpidem) and triazolopyridazines (Cl-218872) barely potentiated Cl^- currents with the $\beta2\gamma2$ subtype, and their levels of potentiation did not improve even at higher drug concentrations (i.e., 10 μM). It appears that the $\beta2\gamma2$ subtype possesses a benzodiazepine site, but its functional characteristics are certainly different from those of the subtypes containing $\alpha1$ and $\gamma2$ subunits.

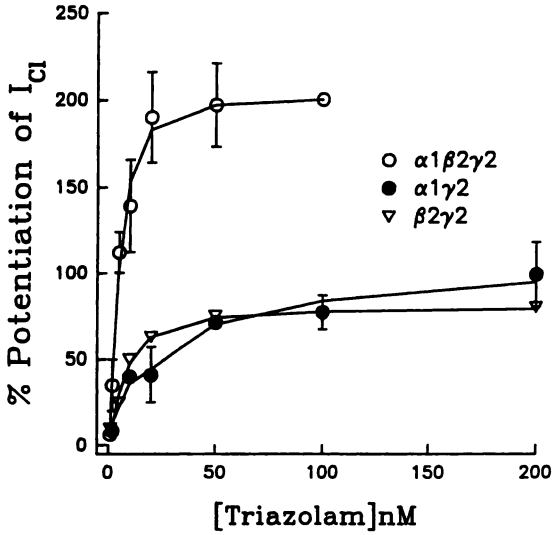

Fig. 3. Dose-response profiles for triazolam potentiation of Cl^- currents with the $\alpha1\beta2\gamma2$, $\alpha1\gamma2$, and $\beta2\gamma2$ subtypes of $GABA_A$ receptors. The net increases in Cl^- currents produced by triazolam at the indicated concentrations were plotted and fitted to the equation given in the text. The data represent means ± standard errors from three separate measurements.

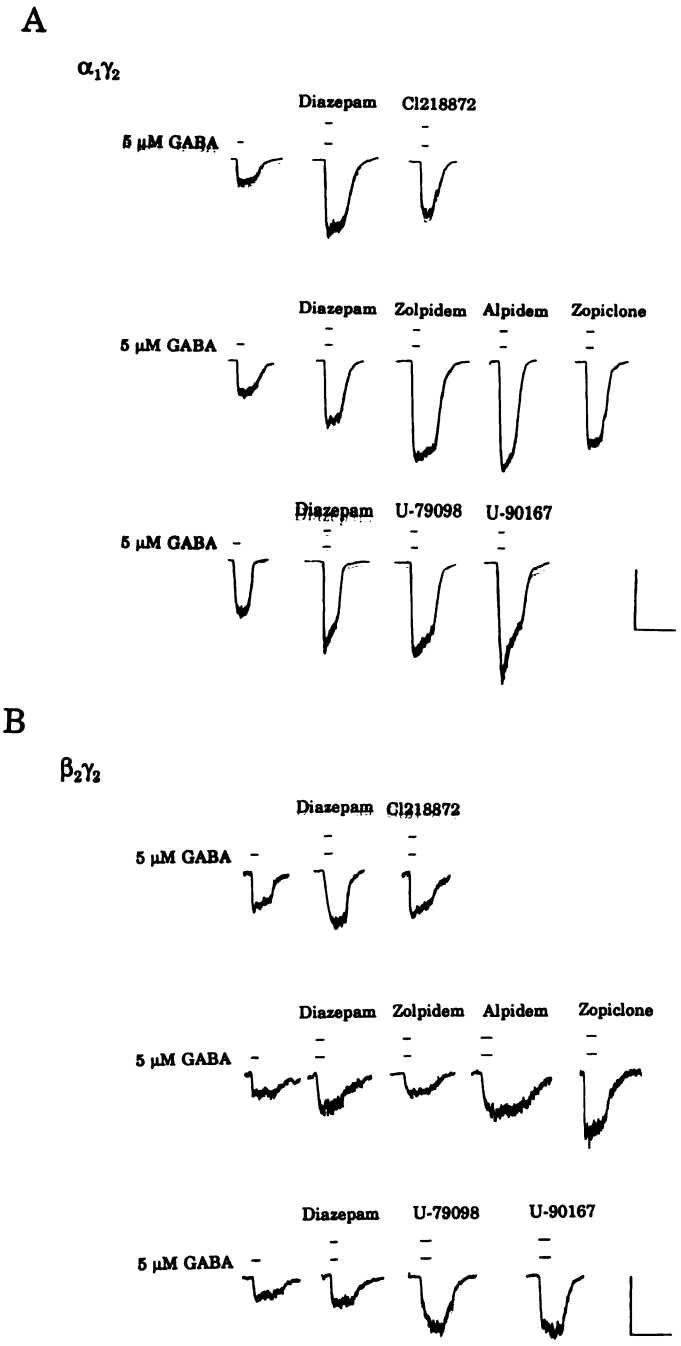

Fig. 4. *Traces* showing the effects of various benzodiazepine site agonists on GABA-mediated Cl^- currents with the $\alpha1\gamma2$ and $\beta2\gamma2$ subtypes of $GABA_A$ receptors. Cl^- currents were induced with GABA at 5 μM, in the absence or presence of the drugs at 5 μM, in A293 cells expressing the $\alpha1\gamma2$ (A) or $\beta2\gamma2$ (B) subtypes of $GABA_A$ receptors. When more than one drug was tested in the same patch, the patch was washed until the GABA response was restored to the original level. *Scale in the lower right corner, vertical bar*, 500 pA (A) or 200 pA (B); *horizontal bar*, 30 sec.

Ro 15-1788 has been established as a selective antagonist for benzodiazepine sites in the $\alpha1\beta2\gamma2$ subtype and in neuronal $GABA_A$ receptors (3, 26). Fig. 5 shows that the antagonist also blocked potentiation of GABA-induced Cl^- currents by alpidem

TABLE 1

Degrees of potentiation of GABA-induced Cl^- currents with the α1β2γ2, α1γ2, and β2γ2 subtypes of cloned $GABA_A$ receptors by various benzodiazepine site agonists

GABA (5 μM)-induced Cl^- currents were measured, using the whole-cell configuration of the patch-clamp technique, in A293 cells expressing the various subtypes of $GABA_A$ receptors. The holding potential was −60 mV under a symmetrical Cl^- gradient. The degrees of potentiation by a test drug represent the net increase of the peak Cl^- current in the presence of the drug at 5 μM, after normalization to Cl^- currents observed with 5 μM GABA alone. The data represent the mean ± standard error from at least five separate measurements.

	Potentiation of GABA-induced Cl^- currents		
	α1β2γ2	α1γ2	β2γ2
		%	
Diazepam	95 ± 23	110 ± 31	53 ± 18
Triazolam	196 ± 25	117 ± 43	71 ± 16
Alpidem	201 ± 33	171 ± 42	38 ± 29
Zolpidem	127 ± 27	154 ± 25	6 ± 6
Cl-218872	98 ± 24	104 ± 31	0 ± 5
Zopiclone	117 ± 25	154 ± 25	114 ± 50
U-79098	254 ± 42	139 ± 32	95 ± 40
U-90167	220 ± 25	112 ± 22	120 ± 22

and triazolam with the α1γ2 subtype and abolished the similar effect of U-90167 on the β2γ2 subtype. This indicates that the α1β2γ2, α1γ2, and β2γ2 subtypes share core overlapping structural domains in their benzodiazepine binding sites.

Discussion

The major point of this study is that the β2γ2 subtype of $GABA_A$ receptor has a benzodiazepine site. Using agonists of diverse chemical structures, we showed that the benzodiazepine site in the β2γ2 subtype displayed differential responses to agonistic ligands; the imidazoquinoxaline U-79098 and the diimidazoquinazoline U-90167 acted as full agonists with the β2γ2 subtype, as with the α1γ2 and α1β2γ2 subtypes, whereas the imidazopyridines alpidem and zolpidem and the triazolopyridazine Cl-218872, which are full agonists with the latter subtypes, produced no or marginal potentiation of GABA currents with the β2γ2 subtype. Classical benzodiazepines (diazepam and triazolam) potentiated Cl^- currents with the β2γ2 subtype to about 30–50% of those observed with the α1γ2 and the α1β2γ2 subtypes.

The attenuated (even absent) responses of the β2γ2 subtype to the imidazopyridines and the triazolopyridazine could be due to changes in their binding affinities and/or in their maximal efficacies. In this study we were not able to measure their binding affinities because of low levels of receptor expression in the cloned cells. However, as the drug concentration was changed from 5 to 10 μM, we observed no noticeable changes in the drug responses. It appears that their low levels of Cl^- current potentiation are not due to their limited occupancy of the binding site but are probably due to their reduced intrinsic efficacy with the β2γ2 subtype. This seems to be supported by our observations with triazolam. The maximal efficacy of triazolam with the β2γ2 subtype was about 30% of that observed with the α1β2γ2 subtype, and its ED_{50} value was not noticeably different from that with the α1β2γ2 subtype. It appears that the pharmacophore responsible for the agonistic activity of selective compounds with the β2γ2 subtype was altered, compared with the α1β2γ2 subtype.

Our observations in this study yield several interesting points about the structural aspects of the benzodiazepine site on

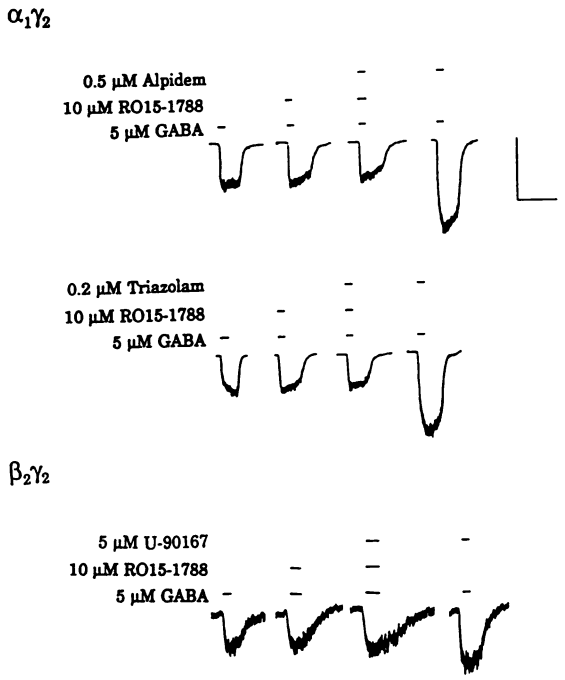

Fig. 5. Effect of Ro 15–1788 on potentiation of Cl^- currents by triazolam and alpidem with the α1γ2 subtype and by U-90167 with the β2γ2 subtype of $GABA_A$ receptors. *Upper*, Cl^- currents were induced with 5 μM GABA in the presence of alpidem (0.5 μM), Ro 15–1788 (10 μM), or a combination of the two drugs, with the α1γ2 subtype. *Middle*, similar experiments with triazolam (0.2 μM), Ro 15–1788 (10 μM), or the combination of the two, with the α1γ2 subtype. *Lower*, similar competition study with U-90167 (5 μM) and Ro 15–1788 (10 μM), with the β2γ2 subtype. Application of each drug was followed by extensive washing of the patch. When the original GABA response was restored, we proceeded with application of another drug. *Scale in the upper right corner*, *vertical bar*, 500 pA (*upper* and *middle*) or 200 pA (*lower*); *horizontal bar*, 30 sec.

cloned $GABA_A$ receptors. The similarity of the responses of various agonists with the α1γ2 and the α1β2γ2 subtypes is in agreement with the earlier report that the α1 and γ2 subunits may provide nearly all the necessary structural domains for the benzodiazepine site (17). At the same time, the expression of a benzodiazepine site in the β2γ2 subtype, albeit different from that in the α1β2γ2 and α1γ2 subtypes, shows its versatility and indicates that the β2 subunit can substitute for the α1 subunit in the formation of a benzodiazepine site. β2, however, cannot substitute for γ2, as shown by the insensitivity of α1β2 to benzodiazepines.

The most interesting finding regarding the structural aspects of the benzodiazepine site is the selective insensitivity of the β2γ2 subtype to the imidazopyridines and the triazolopyridazine, so called type 1-selective drugs (α1-selective, compared with subtypes containing α2 or α3) (10). One simple explanation could be that the response to the type 1 ligands requires domains specific for α1, whereas the nonselective ligands use different sets of interacting domains that may be supplied by β2 and by γ2. We propose that the benzodiazepine site in the α1β2γ2 subtype, which may represent the majority of native $GABA_A$ receptors, is located at the interface of the α1 and γ2 subunits, consisting of structural domains primarily contributed by the two subunits.

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