0026-895X/94/050957-07$03.00/0

MOLECULAR PHARMACOLOGY, 46:957-963, 1994.

Molecular Pharmacology of γ-Aminobutyric Acid Type A Receptor Agonists and Partial Agonists in Oocytes Injected with Different α, β, and γ Receptor Subunit Combinations

BJARKE EBERT, KEITH A. WAFFORD, PAUL J. WHITING, POVL KROGSGAARD-LARSEN, and JOHN A. KEMP

PharmaBiotec Research Centre, Department of Medicinal Chemistry, The Royal Danish School of Pharmacy, DK-2100 Copenhagen, Denmark (B.E., P.K.-L.), Department of Pharmacology and Biochemistry, Neuroscience Research Centre, Merck Sharp and Dohme Research Laboratories, Harlow, Essex CM20 2QR, England (K.A.W., P.J.W.), and Central Nervous System Department, Pharma Division, Preclinical Research, Hoffmann-La Roche Ltd., CH-4002 Basel, Switzerland (J.A.K.)

Received May 24, 1994; Accepted August 1, 1994

SUMMARY

Using systematic combination of α1, α3, and α5 with β1, β2, and β3, together with γ1, γ2, and γ3, we have investigated the contributions of the various α, β, and γ subunits to the pharmacology of γ-aminobutyric acid (GABA)$_A$ agonists. We have characterized GABA, (*RS*)-dihydromuscimol, piperidine-4-sulfonic acid, and 4,5,6,7-tetrahydroisoxazolo[5,4-*c*]pyridin-3-ol with recombinant human GABA$_A$ receptors expressed in *Xenopus* oocytes. Our observations indicate that the α subunit is the major determinant of efficay for partial GABA$_A$ agonists. When α1 and α3 or α1 and α5 are coexpressed, the α1 subunit determines the maximum efficacy, whereas the affinity is determined by the entire combination of subunits. Thus, the results of the present study demonstrate that the pharmacology of GABA$_A$ agonists is dependent on the subunit composition of the GABA$_A$ receptor complex. Functional GABA$_A$ receptors containing two different α subunits show pharmacological profiles distinctly different from those of receptors containing a single α subtype, indicating that two different α subunits can be coexpressed in one functional GABA$_A$ receptor complex.

The GABA$_A$ receptor complex is composed of a hetero-oligomeric membrane protein that forms a chloride-permeable ion channel. The functional GABA$_A$ complex is formed by a combination of α, β, and γ or δ subunit proteins, most of which exist in several variants and alternatively spliced versions (1–11). The molecular diversity of GABA$_A$ receptors is also reflected in the pharmacologically different actions of allosteric modulators of GABA$_A$ receptor function (12, 13). The subunit composition-dependent pharmacology of benzodiazepines and β-carbolines at GABA$_A$ receptor complexes has been characterized in detail, and it has been concluded that the α and γ subunits both contribute to the affinity and agonist/antagonist/inverse agonist effects of these compounds (10, 11, 14–16). In contrast to this, only little is known regarding the subunit composition-dependent pharmacology of compounds acting directly at the GABA$_A$ receptor recognition site. We have now attempted to carry out a systematic study to examine the roles played by different α, β, and γ subunits in determining the affinity and efficacy of four compounds acting at the GABA$_A$ agonist binding site.

This work was supported by grants from the Lundbeck Foundation and the Danish State Biotechnology Programme (1991–1995).

We have previously characterized the high affinity, GABA$_A$ receptor, agonists DHM (17), P4S (18, 19), and THIP (20, 21) in receptor binding assays and with electrophysiological recordings from cat spinal cord neurons. Experiments in which [^{3}H] diazepam binding was stimulated with GABA$_A$ agonists have shown that P4S may not be able to activate the receptor to the same extent as muscimol or GABA (22), suggesting that P4S may be a partial agonist. However, this finding has not been confirmed in other assays. We have now characterized the pharmacology of GABA, DHM, THIP, and P4S at functional GABA$_A$ receptors expressed in *Xenopus* oocytes. By systematic variation of the subunit composition, we have tried to map the influence of different subunits on the efficacy and affinity of the aforementioned compounds.

Materials and Methods

cDNAs. cDNAs encoding human α1, α3, α5, β1, β2, β3, γ1, and γ2 subunits have been described elsewhere (15, 16, 23). Cloning and sequencing of cDNAs encoding human γ3 subunits will be described elsewhere.[1]

[1] Whiting *et al.*, unpublished manuscript.

ABBREVIATIONS: GABA, γ-aminobutyric acid; DHM, (*RS*)-dihydromuscimol; P4S, piperidine-4-sulfonic acid; THIP, 4,5,6,7-tetrahydroisoxazolo-[5,4-*c*]pyridin-3-ol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Oocyte expression. *Xenopus* oocytes were removed from anesthetized frogs and manually defolliculated with fine forceps. After mild collagenase treatment (type IA, 0.5 mg/ml, for 10 min) to remove follicle cells, the oocyte nuclei were then directly injected with 10–20 nl of injection buffer (88 mM NaCl, 1 mM KCl, 15 mM HEPES, pH 7.0; filtered through nitrocellulose) containing different combinations of human $GABA_A$ subunit cDNAs (6 ng/ml) engineered into the expression vector pCDM8 or pcDNAAmp. After incubation for 24 hr, oocytes were placed in a 50-μl bath and perfused with modified Barth's medium [88 mM NaCl, 1 mM KCl, 10 mM HEPES, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 0.91 mM $CaCl_2$, 2.4 mM $NaHCO_3$, pH 7.5]. Cells were impaled with two 1–3-MΩ electrodes containing 2 M KCl and were voltage clamped between −40 and −70 mV. The cells were continuously perfused with saline at 4–6 ml/min, and drugs were applied in the perfusate. GABA or $GABA_A$ agonists were applied until the peak of the response was observed, usually 30 sec or less. At least 3 min of wash time were allowed between each agonist application, to prevent desensitization. Data from each oocyte were analyzed with respect to the maximum response, relative to either the plateau level of a full GABA concentration-response curve or the response to 3 mM GABA (no difference). Concentration-response curves were calculated using a nonlinear, least-squares, fitting program with the equation $f(x) = B_{max}/[1 + (EC_{50}/x)^n]$, where x is the drug concentration, EC_{50} is the concentration of drug eliciting a half-maximal response, and n is the Hill coefficient. P4S, THIP, and DHM were synthesized as described previously (19, 24, 25). Additional P4S was obtained from Tocris Neuramin, and all other compounds were obtained from Sigma Biochemicals or Research Biochemicals. The computer program GraFit 3.0 (Erithacus Software, Staines, UK) was used to analyze and plot data.

Results

General. GABA, DHM, THIP, and P4S (Fig. 1) were all characterized using $GABA_A$ receptors in which the α subunit (α1, α3, or α5) and the β subunit (β1, β2, or β3) were varied in the presence of γ2. Concentration-response curves for each single oocyte were analyzed and the maximum response for GABA was established. Maximum responses for other agonists were always determined relative to the maximum GABA response in the same oocyte. Results from these studies based on systematic variation of the α and β subunits are shown in Figs. 2 and 3 and Table 1.

Role of the α subunit. Affinity for GABA was clearly dependent on the type of α subunit present in the receptor. For most combinations, the affinity was between 10 and 30 μM; however, α3-containing receptors, particularly when combined with β1, exhibited a lower affinity (Fig. 3A). Also, α5-containing receptors generally had a slightly higher affinity for GABA than did α1- or α3-containing receptors. DHM was a full agonist at receptors containing all subunit combinations and, as can be seen in Fig. 3A, had a pattern of subunit-dependent affinity very similar to that of GABA. The same general pattern of affinities was seen for all compounds, with the highest affinity for α5β3γ2 and the lowest for α3β1γ2. Generally, GABA gave the largest variation in affinity, showing a 70-fold

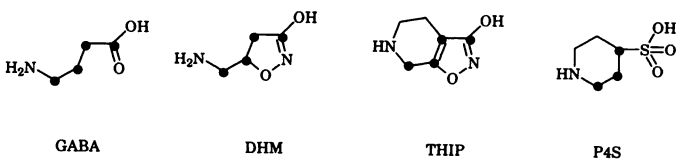

Fig. 1. Structures of GABA and the $GABA_A$ receptor agonists DHM, THIP, and P4S.

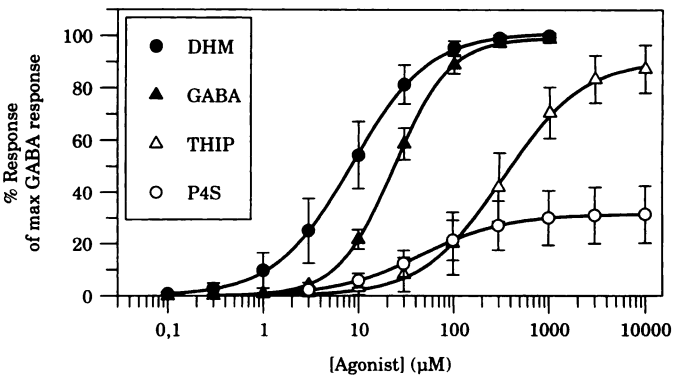

Fig. 2. Concentration-response curves for GABA, THIP, DHM, and P4S in oocytes injected with α1β2γ2. Data points are mean values ± standard errors from at least four individual oocytes. *Curves* were fitted using the equation described in Materials and Methods. Parameter values and standard errors are shown in Table 1.

difference between α5β3γ2 and α3β1γ2, compared with 15-fold for DHM, 12-fold for THIP, and 8-fold for P4S.

The α subunits showed a marked effect on the degree of efficacy of partial agonists (Fig. 3B). THIP and P4S both showed levels of efficacy dependent on the type of α subunit present. P4S was a partial agonist with approximately 32% efficacy with $GABA_A$ receptors containing α1 but with 70–90% efficacy with oocytes expressing α3βxγ2 and approximately 90% efficacy with receptors containing α5. Similarly, THIP showed a much lower degree of efficacy with receptors containing α3 (50%), compared with those containing α1 (70–78%) or α5 (81–100%) (Fig. 3B). There were, however, minor deviations from this generalization. For example, the maximum efficacy of P4S dropped to 49% with α5β1γ2 receptors, compared with 90% for α5β2γ2 or α5β3γ2 receptors.

Role of the β subunit. In the presence of α1, different β subunits had very little effect on either affinity or efficacy of $GABA_A$ agonists (Fig. 3). In the case of α5, a slight increase in affinity was observed for most agonists, in the order β3 > β2 > β1. Also, the α3β1γ2 combination produced receptors with particularly low affinity for all agonists. Different β subunits did not affect efficacy, with the exception of that of P4S, which was a full agonist at α3β2γ2 receptors (99% efficacy), compared with 61% and 75% efficacy at α3β1γ2 and α3β2γ2 receptors, respectively. P4S was also a partial agonist at α5β1γ2 receptors (49% efficacy), compared with 90% and 92% efficacy at α5β2γ2- and α5β3γ2-containing receptors, respectively. Overall, changes in the type of β subunit produced smaller effects on agonist pharmacology than did changes in the α subunit.

Role of the γ subunit. To further investigate the influence of the subunit composition on the affinity and efficacy of $GABA_A$ agonists, we also varied the γ subunit in two receptor complexes with fixed αβ compositions. Because GABA and DHM were both full agonists with the tested combinations of α and β subunits in the presence of γ2, we decided to use GABA, THIP, and P4S in these experiments.

All three agonists showed highest affinities using combinations containing the γ3 subunit (Fig. 4; Table 1), with decreasing affinity when γ2 and γ1 were incorporated, although in the presence of α3β1 there was a much smaller change in affinity with different γ subunits. The partial agonists THIP and P4S both showed significant reductions in efficacy with γ1-containing receptors, whereas both compounds exhibited increased

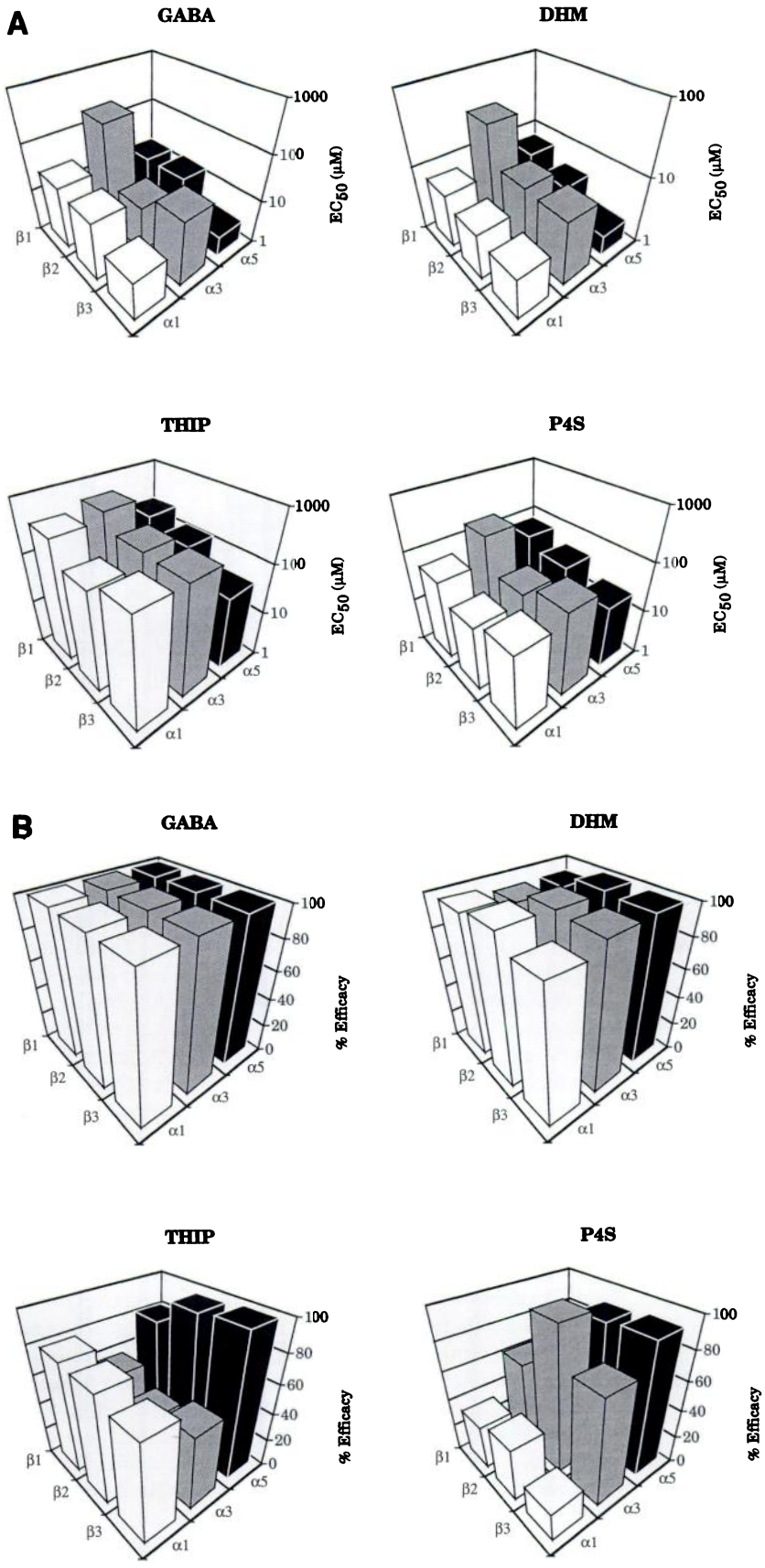

Fig. 3. GABA agonist properties at GABA$_A$ receptor subunit combinations containing different α and β subunits. A, EC_{50} values for GABA, THIP, DHM, and P4S in oocytes injected with different $\alpha\beta$ subunit combinations together with γ2. *x-Axis*, α1–3; *y-axis*, β1–3; *z-axis*, EC_{50} values. Actual values and standard errors are shown in Table 1. B, Agonist efficacies, relative to that of GABA (100%), for GABA, THIP, DHM, and P4S in oocytes injected with different $\alpha\beta$ subunit combinations together with γ2. *x-Axis*, α1–3; *y-axis*, β1–3; *z-axis*, maximum efficacy (percentage of maximum GABA response). Actual values and standard errors are shown in Table 1.

TABLE 1

Pharmacology of $GABA_A$ agonists in oocytes injected with different subunit combinations

EC_{50} values and degrees of maximal efficacy, relative to GABA, of GABA, DHM, THIP, and P4S in oocytes expressing different combinations of α, β, and γ subunits are shown. Values represent means ± standard errors of at least four experiments.

	GABA		DHM		THIP		P4S	
	Maximal efficacy	EC_{50}	Maximal efficacy	EC_{50}	Maximal efficacy	EC_{50}	Maximal efficacy	EC_{50}
	%	μM	%	μM	%	μM	%	μM
$\alpha1\beta1\gamma2$	100	25 ± 4	96 ± 4	7 ± 2	78 ± 6	358 ± 60	32 ± 11	52 ± 5
$\alpha1\beta2\gamma2$	100	20 ± 3	100 ± 0	5 ± 1	76 ± 4	143 ± 15	38 ± 5	25 ± 4
$\alpha1\beta3\gamma2$	100	8 ± 2	92 ± 4	4 ± 1	70 ± 3	238 ± 43	21 ± 6	44 ± 8
$\alpha3\beta1\gamma2$	100	208 ± 72	92 ± 3	33 ± 4	56 ± 6	499 ± 43	61 ± 3	165 ± 24
$\alpha3\beta2\gamma2$	100	11 ± 2	99 ± 2	10 ± 6	44 ± 4	246 ± 41	99 ± 1	36 ± 8
$\alpha3\beta3\gamma2$	100	28 ± 4	98 ± 2	9 ± 1	53 ± 6	233 ± 32	75 ± 6	65 ± 7
$\alpha5\beta1\gamma2$	100	15 ± 3	93 ± 4	7 ± 1	81 ± 2	218 ± 59	49 ± 9	74 ± 13
$\alpha5\beta2\gamma2$	100	16 ± 2	101 ± 1	5 ± 1	97 ± 5	129 ± 25	90 ± 6	46 ± 3
$\alpha5\beta3\gamma2$	100	3 ± 1	99 ± 1	2 ± 1	99 ± 1	40 ± 9	92 ± 7	22 ± 4
$\alpha1\alpha3\beta2\gamma2$	100	26 ± 5					42 ± 4	145 ± 6
$\alpha1\alpha5\beta2\gamma2$	100	18 ± 7					32 ± 5	76 ± 13
$\alpha3\beta1\gamma1$	100	114 ± 34			32 ± 9	385 ± 25	63 ± 4	300 ± 112
$\alpha3\beta1\gamma2$	100	208 ± 71			56 ± 6	499 ± 42	61 ± 3	165 ± 24
$\alpha3\beta1\gamma3$	100	32 ± 6			73 ± 7	277 ± 63	82 ± 1	41 ± 2
$\alpha5\beta3\gamma1$	100	24 ± 3			68 ± 5	339 ± 45	24 ± 4	166 ± 2
$\alpha5\beta3\gamma2$	100	3 ± 1			99 ± 1	40 ± 9	92 ± 7	22 ± 3
$\alpha5\beta3\gamma3$	100	2 ± 1			93 ± 5	28 ± 10	86 ± 6	16 ± 4

efficacy with $\gamma2$- and $\gamma3$-containing receptors. The γ subunit thus seems to be a co-determinant for efficacy as well as affinity of agonists in recombinant $GABA_A$ receptors.

Receptors containing two different α subunits. To examine more closely the effects of α subunits on the pharmacology of GABA and P4S, we coinjected $\alpha1$ (at which P4S is a partial agonist) and $\alpha3$ or $\alpha5$ (at which P4S is a full agonist) in combination with $\beta2\gamma2$. The results (Fig. 5; Table 1) showed that the affinity of GABA was unaltered by the presence of two different α subunits ($\alpha1\alpha3$ or $\alpha1\alpha5$) in the same receptor complex, being most similar to that for $\alpha1\beta2\gamma2$. The maximum response to P4S was not significantly different from the maximum response with $\alpha1\beta2\gamma2$ receptors but was significantly lower than that with $\alpha3\beta2\gamma2$ or $\alpha5\beta2\gamma2$ $GABA_A$ receptors. The affinity of P4S for the double-α subunit hetero-oligomers was significantly lower than its affinity for combinations containing identical α subunits, for example, 145 ± 6 μM for $\alpha1\alpha3\beta2\gamma2$ and 76 ± 13 μM for $\alpha1\alpha5\beta2\gamma2$, compared with 36 ± 8 μM for $\alpha3\beta2\gamma2$ and 25 ± 4 μM for $\alpha1\beta2\gamma2$. Thus, the assembly of receptors containing two different α subunits results in a novel pharmacology with respect to $GABA_A$ agonists.

Discussion

Several studies have addressed the question of the location of the agonist binding site(s) in the $GABA_A$ receptor complex (26–28). In early studies, [^{3}H]muscimol was shown to label a receptor protein corresponding to the β subunit (29); however, recent experiments have also identified [^{3}H]muscimol photoaffinity labeling on protein fragments that correspond to an α subunit (30). Homomeric α, β, and γ receptors all assemble with low efficiency and respond to GABA, suggesting the presence of GABA binding site(s) on all subunits; however, experiments comparing $\alpha\beta$, $\beta\gamma$, and $\alpha\gamma$ combinations show robust expression only for $\alpha\beta$ and $\beta\gamma$. Futhermore, no currents have been observed for $\alpha\gamma$ combinations,[2] suggesting that the β subunit of the $GABA_A$ receptor complex is a component of critical importance for GABA binding. Similarly, site-directed mutagenesis studies have identifed domains of the β subunit that are critical for activation by GABA but that do not affect pentobarbital activation of the receptor (31). Another study has also identified amino acid residues on the α subunit that contribute to GABA agonist and antagonist affinity (32), suggesting that GABA agonist binding is clearly not restricted to a single type of receptor subunit.

By systematic variation of the α and β subunits in the presence of $\gamma2$, we observed that both the α and β subunits can contribute to the affinity of $GABA_A$ agonists. This is most convincingly seen in Fig. 3A, where the variation in EC_{50} values was influenced by different forms of α and β subunits. The same general pattern of affinities was seen for all of the compounds, suggesting that these $GABA_A$ agonists interact with the same residues on individual receptor combinations. In light of these observations, it is tempting to speculate that the agonist site of the $GABA_A$ receptor complex is formed at the interface between the α and β subunits. This idea, however, is not supported by data comparing receptors with different γ subunits. The γ subunit was also shown to contribute significantly to the affinity of $GABA_A$ agonists (Fig. 4), suggesting that affinity is influenced by α, β, and γ subunits. Other studies have compared the EC_{50} values of GABA for different $GABA_A$ receptor combinations expressed in *Xenopus* oocytes. The findings here are generally in agreement with those other studies, with the exception of results with the $\alpha3\beta2\gamma2$ combination, which had a higher affinity for GABA than reported in other studies (26, 33). The explanation for this is currently unclear but one possibility is a species difference, because other studies used rat cDNAs, compared with human cDNAs used in this study. From the data depicted in Fig. 4, it is clear that agonist efficacy is determined primarily by the α subunit and is relatively unaffected by variation of the β subunit. Thus, for receptor combinations containing $\alpha1$ P4S was a partial agonist with an efficacy of approximately 30%, compared with GABA, whereas P4S was almost a full agonist for combinations with

[2] K. A. Wafford, unpublished observations.

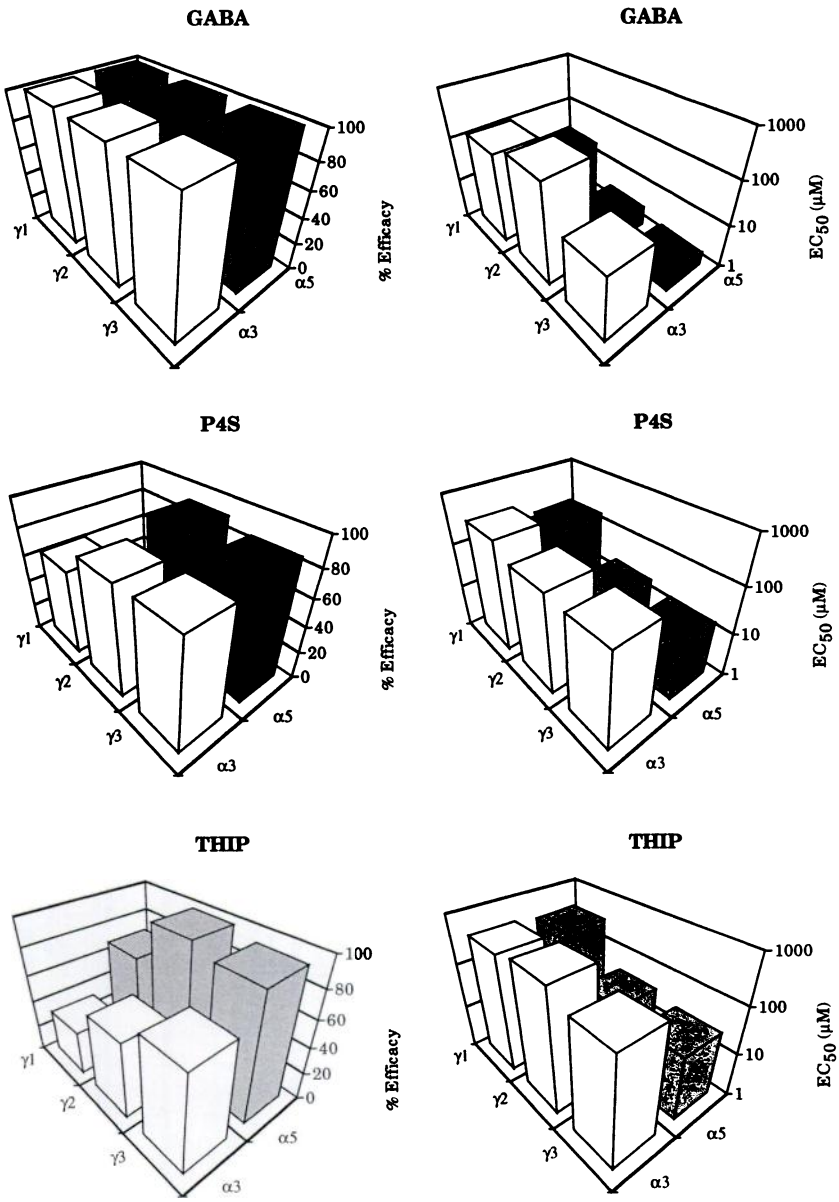

Fig. 4. GABA agonist properties at GABA$_A$ receptor combinations containing different γ subunits. Maximum efficacies (*left*) and EC_{50} values (*right*) for GABA (*top*), P4S (*middle*), and THIP (*bottom*) in oocytes injected with different γ subunits in the presence of α3β1 (*white bars*) or α5β3 (*gray bars*) are shown. Data are mean values from at least four individual oocytes. Actual values and standard errors are shown in Table 1.

α3 or α5. Quite interestingly, THIP was also a partial agonist with α1βxγ2 and α3βxγ2 subunit combinations but, in contrast to P4S, THIP had a higher degree of efficacy with α1βxγ2 and α5βxγ2 than α3βxγ2 combinations. Previous studies have compared these two compounds at the ρ1 subunit, as well as at α5β1 combinations (34). Both compounds were inactive as agonists at the former type of recombinant GABA$_A$ receptor, but THIP was a full agonist and P4S a partial agonist at α5β1 receptors (34). In another study, both compounds were shown to be antagonists at the homo-oligomeric ρ1 GABA$_A$ receptor found exclusively in retina (35).

The relationship between structure and degree of efficacy of GABA$_A$ agonists was different from the structure-affinity relationship for the same series of compounds, suggesting that efficacy is dependent on the structure of the agonist as well as the subunit combination. The observation of different determinants for agonist efficacy and for affinity is supported by recent evidence that mutagenesis of a single amino acid in the γ2 subunit changes the efficacy of certain benzodiazepines without changing their affinity. Thus, it is not possible to predict the efficacy of an agonist at a certain recombinant GABA$_A$ receptor complex based on results from other subunit combinations. The effects of variation of the γ subunit clearly indicate that this subunit also can contribute to the efficacy of partial GABA$_A$ agonists, and agonist efficacy is thus determined primarily by the α and γ subunits. From these data we cannot conclude that the binding site is located at the interface between two of the subunits. It seems more likely that the binding site is located on one subunit and that the interaction between all subunits in the receptor complex determines the three-dimensional structure of the binding site and therefore the interaction with and effects of ligands.

The *Xenopus* oocytes and the application system used do not allow fast events, like desensitization, to be detected or quantified. Therefore, it is likely that the determined maximum responses to the full agonists GABA and DHM are underestimated and that the described pharmacology may represent the pharmacology of the desensitized state of the receptors. If this is the case, then the result would be affinities slightly lower than those measured in systems where more rapid perfusion is

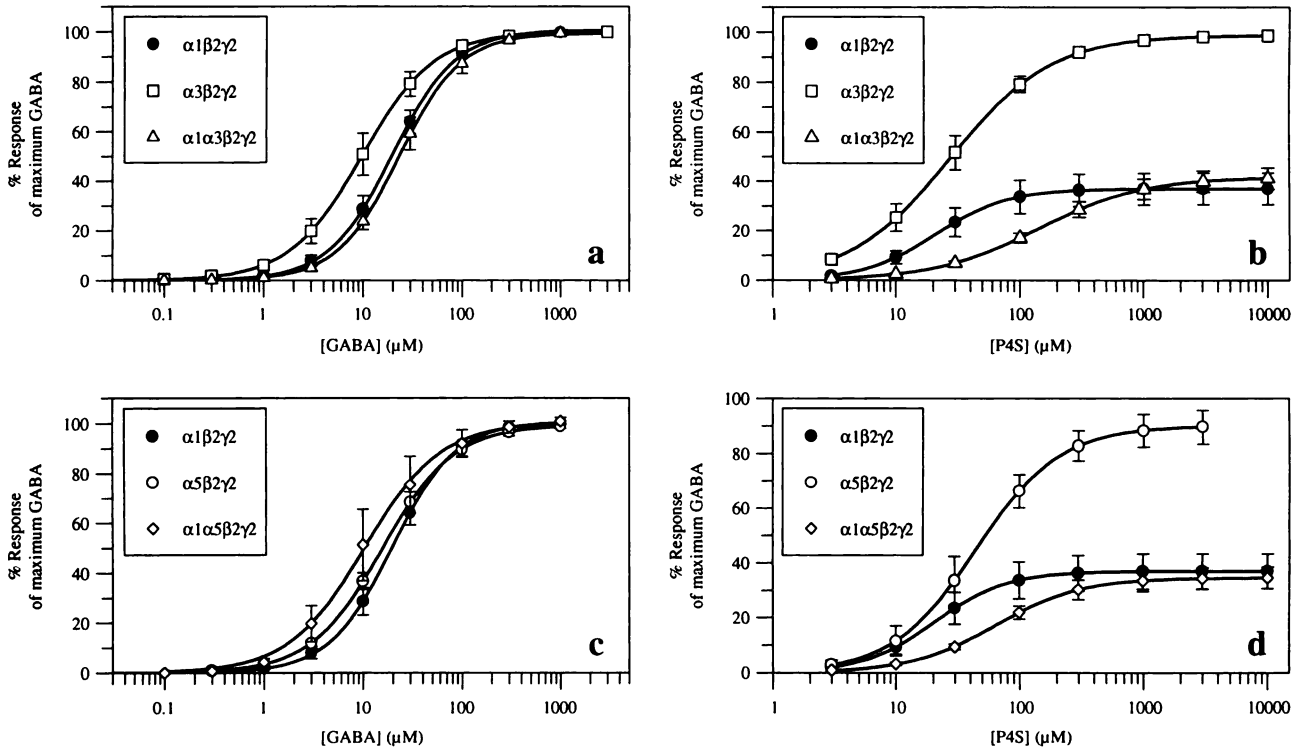

Fig. 5. Properties of $GABA_A$ receptors containing two different types of α subunit. Concentration-response curves for GABA (a and c) and P4S (b and d) in oocytes injected with $\alpha1\beta2\gamma2$, $\alpha3\beta2\gamma2$, $\alpha5\beta2\gamma2$, $\alpha1\alpha3\beta2\gamma2$, or $\alpha1\alpha5\beta2\gamma2$ are shown. Data points are mean values ± standard errors. Parameter values and standard errors are shown in Table 1.

used. Effects on the EC_{50} values, however, are probably small, because results from similar experiments with transfected cells, where rapid application reduces the effects of desensitization, show EC_{50} values similar to those determined in oocytes (37). Similarly, it is possible that differences in desensitization of different subunit combinations may account for some of the observed differences in partial agonist efficacy. If the maximum response to the full agonist GABA is underestimated, P4S and THIP would appear as less efficacious partial agonists. Additional patch-clamp studies on transfected cells will be required to determine whether there are any effects of receptor kinetics on partial agonist efficacy.

In a previous study using subunit combinations of two or more different α and β subunits, it was proposed that receptors that contain more than one α subunit may exist (38). A recent study has also shown that $\alpha1$ and $\alpha3$ subunits coassemble with $\beta2\gamma2$ to form a single receptor with an intermediate GABA affinity and properties distinct from those of receptors consisting of $\alpha1\beta2\gamma2$ or $\alpha3\beta2\gamma2$ (39). The present experiments were designed to examine the properties of the receptor produced by coexpression of two different α subunits ($\alpha1\alpha3$ or $\alpha1\alpha5$) in one receptor complex. The resulting $GABA_A$ receptor showed a pharmacological profile distinctly different from that seen with $\alpha x\beta2\gamma2$ receptors containing a single α subunit. The maximum response to P4S with $\alpha1\alpha3\beta2\gamma2$- or $\alpha1\alpha5\beta2\gamma2$-containing receptors was clearly more like that with $\alpha1\beta2\gamma2$ than $\alpha3\beta2\gamma2$ or $\alpha5\beta2\gamma2$ receptors. The affinity for P4S was, however, lower than that observed using any of the combinations containing only a single α subunit, indicating that the $GABA_A$ receptors formed are different from the $\alpha x\beta2\gamma2$ receptors. These results are consistent with data on immunoprecipitated receptors, using subunit-specific antibodies to establish which subunits are coexpressed in the same receptor (40). Evidence suggests that, although the major $GABA_A$ receptor populations in the brain contain a single type of α subunit, there apparently are small populations that contain both $\alpha1$ and $\alpha3$, $\alpha1$ and $\alpha2$, $\alpha2$ and $\alpha3$, and $\alpha1$ and $\alpha6$ (38, 40–41). It is interesting to note that these combinations can form $GABA_A$ receptor complexes with pharmacological properties distinctly different from those containing a single α subunit. Because the $GABA_A$ receptor is probably a pentameric structure, it is also possible that other receptors containing multiple β or γ as well as α subunits may form as subpopulations in the brain, with different pharmacological properties. These results therefore add yet another layer of complexity to the heterogeneity of $GABA_A$ receptor channels.

Acknowledgments

The secretarial assistance of Mrs. Anne Nordly is gratefully acknowledged.

References

1. Burt, D. R., and G. L. Kamatchi. $GABA_A$ receptor subtypes: from pharmacology to molecular biology. *FASEB J.* **5**:2916–2923 (1991).
2. Wisden, W., and P. H. Seeburg. $GABA_A$ receptor channels: from subunits to functional entities. *Curr. Opin. Neurobiol.* **2**:263–269 (1992).
3. Pritchett, D. B., H. Lüddens, and P. H. Seeburg. Type I and type II $GABA_A$-benzodiazepine receptors produced in transfected cells. *Science (Washington D. C.)* **245**:1389–1392 (1989).
4. Pritchett, D. B., and P. H. Seeburg. γ-Aminobutyric $acid_A$ receptor $\alpha5$-subunit creates novel type II benzodiazepine receptor pharmacology. *J. Neurochem.* **54**:1802–1804 (1990).
5. Lüddens, H., D. B. Pritchett, M. Köhler, I. Killish, K. Keinanen, H. Monyer, R. Sprengel, and P. H. Seeburg. Cerebellar $GABA_A$ receptor selective for a behavioural alcohol antagonist. *Nature (Lond.)* **346**:648–651 (1990).
6. Wisden, W., A. Herb, H. Wieland, K. Keinanen, H. Lüddens, and P. H. Seeburg. Cloning, pharmacological characteristics and expression pattern of the rat $GABA_A$ receptor $\alpha4$ subunit. *FEBS Lett.* **289**:227–230 (1991).
7. Pritchett, D. B., H. Sontheimer, B. D. Shivers, S. Ymer, H. Kettenmann, P. R. Schofield, and P. H. Seeburg. Importance of a novel $GABA_A$ receptor

subunit for benzodiazepine pharmacology. *Nature (Lond.)* **338**:582–585 (1989).
8. Ymer, S., A. Draguhn, W. Wisden, P. Werner, K. Keinanen, P. R. Schofield, R. Sprengel, D. B. Pritchett, and P. H. Seeburg. Structural and functional characterization of the γ1 subunit of GABA$_A$/benzodiazepine receptors. *EMBO J.* **9**:3261–3267 (1990).
9. Wilson-Shaw, D., M. Robinson, C. Gambarana, R. E. Siegel, and J. M. Sikela. A novel γ subunit of the GABA$_A$ receptor identified using the polymerase chain reaction. *FEBS Lett.* **284**:211–215 (1991).
10. Knoflach, F., T. Rhyner, M. Villa, S. Kellenberger, U. Drescher, P. Malherbe, E. Sigel, and H. Möhler. The γ3-subunit of the GABA$_A$-receptor confers sensitivity to benzodiazepine receptor ligands. *FEBS Lett.* **293**:191–194 (1991).
11. Herb, A., W. Wisden, H. Lüddens, G. Puia, S. Vicini, and P. H. Seeburg. The third γ subunit of the γ-aminobutyric acid type A receptor family. *Proc. Natl. Acad. Sci. USA* **89**:1433–1437 (1992).
12. Barnard, E. A., and E. Costa, eds. *Allosteric Modulation of Amino Acid Receptors: Therapeutic Implications*. Raven Press, New York (1989).
13. Gammill, R. B., and D. B. Carter. Neuronal BZD receptors: new ligands, clones and pharmacology. *Annu. Rev. Med. Chem.* **28**:19–27 (1993).
14. Puia, G., S. Vicini, P. H. Seeburg, and E. Costa. Influence of recombinant γ-aminobutyric acid$_A$ receptor subunit composition on the action of allosteric modulators of γ-aminobutyric acid-gated Cl^- currents. *Mol. Pharmacol.* **39**:691–696 (1991).
15. Wafford, K. A., P. J. Whiting, and J. A. Kemp. Differences in affinity and efficacy of benzodiazepine receptor ligands on recombinant γ-aminobutyric acid type A receptor subtypes. *Mol. Pharmacol.* **43**:240–244 (1993).
16. Wafford, K. A., C. J. Bain, P. J. Whiting, and J. A. Kemp. Functional comparison of the role of γ subunits in recombinant human γ-aminobutyric acid$_A$/benzodiazepine receptors. *Mol. Pharmacol.* **44**:437–442 (1993).
17. Krogsgaard-Larsen, P., H. Hjeds, D. R. Curtis, D. Lodge, and G. A. R. Johnston. Dihydromuscimol, thiomuscimol and related heterocyclic compounds as GABA analogues. *J. Neurochem.* **32**:1717–1724 (1979).
18. Krogsgaard-Larsen, P., A. Snowman, S. C. Lummis, and R. W. Olsen. Characterization of the binding of the GABA agonist [^{3}H]piperidine-4-sulfonic acid to bovine brain synaptic membranes. *J. Neurochem.* **37**:401–409 (1981).
19. Krogsgaard-Larsen, P., E. Falch, A. Schousboe, D. R. Curtis, and D. Lodge. Piperidine-4-sulfonic acid, a new specific GABA agonist. *J. Neurochem.* **34**:756–759 (1980).
20. Krogsgaard-Larsen, P., G. A. R. Johnston, D. Lodge, and D. R. Curtis. A new class of GABA agonist. *Nature (Lond.)* **268**:53–55 (1977).
21. Krogsgaard-Larsen, P., E. Falch, and A. V. Christensen. Chemistry and pharmacology of the GABA agonists THIP (gaboxadol) and isoguvacine. *Drugs Future* **9**:597–618 (1984).
22. Falch, E., P. Jacobsen, P. Krogsgaard-Larsen, and D. R. Curtis. GABA-mimetic activity and effects on diazepam binding of aminosulfonic acids structurally related to piperidine-4-sulfonic acid. *J. Neurochem.* **44**:68–75 (1985).
23. Hadingham, K. L., P. B. Wingrove, K. A. Wafford, C. J. Bain, J. A. Kemp, K. J. Palmer, A. W. Wilson, A. S. Wilcox, J. Sikela, C. I. Ragan, and P. J. Whiting. The role of the β subunit in determining the pharmacology of human γ-aminobutyric acid type A receptors. *Mol. Pharmacol.* **44**:1211–1218 (1993).
24. Krogsgaard-Larsen, P., L. Nielsen, E. Falch, and D. R. Curtis. GABA agonists: resolution, absolute stereochemistry, and enantioselectivity of (*S*)-(+)- and (*R*)-(−)-dihydromuscimol. *J. Med. Chem.* **28**:1612–1617 (1985).
25. Krogsgaard-Larsen, P. Muscimol analogues. II. Synthesis of some bicyclic 3-isoxazolol zwitterions. *Acta Chem. Scand. B* **3**:584–588 (1977).
26. Verdoorn, T. A., A. Draguhn, S. Ymer, P. H. Seeburg, and B. Sakmann. Functional properties of recombinant rat GABA$_A$ receptors depend upon subunit composition. *Neuron* **4**:919–928 (1990).
27. Olsen, R. W., M. H. Bureau, S. Endo, and G. Smith. The GABA$_A$ receptor family in the mammalian brain. *Neurochem. Res.* **16**:317–325 (1991).
28. Levitan, E. S., P. R. Schofield, D. R. Burt, L. M. Rhee, W. Wisden, M. Köhler, N. Fujita, H. F. Rodriguez, A. Stephenson, M. G. Darlison, E. A. Barnard, and P. H. Seeburg. Structural and functional basis for GABA$_A$ receptor diversity. *Nature (Lond.)* **335**:76–79 (1988).
29. Deng, L., R. W. Ransom, and R. W. Olsen. [^{3}H]Muscimol photolabels the γ-aminobutyric acid receptor binding site on a peptide subunit distinct from that labelled with benzodiazepines. *Biochem. Biophys. Res. Commun.* **138**:1308–1314 (1986).
30. Smith, G. B., and R. W. Olsen. Identification of a [^{3}H]muscimol photoaffinity substrate in GABA-A receptor *alpha* subunit. *Soc. Neurosci. Abstr.* **19**:199.8 (1993).
31. Amin, J., and D. S. Weiss. GABA$_A$ receptor needs two homologous domains of the β-subunit for activation by GABA but not by pentobarbital. *Nature (Lond.)* **366**:565–569 (1993).
32. Sigel, E., R. Baur, S. Kellenberger, and P. Malherbe. Point mutations affecting antagonist affinity and agonist dependent gating of GABA$_A$ receptor channels. *EMBO J.* **11**:2017–2023 (1992).
33. Sigel, E., R. Baur, G. Trube, H. Mohler, and P. Malherbe. The effect of subunit composition of rat brain GABA$_A$ receptors on channel function. *Neuron* **5**:703–711 (1990).
34. Kusama, T., C. E. Spivak, P. Whiting, V. L. Dawson, J. C. Schaeffer, and G. R. Uhl. Pharmacology of GABA ρ1 and GABA α/β receptors expressed in *Xenopus* oocytes and COS cells. *Br. J. Pharmacol.* **109**:200–206 (1993).
35. Woodward, R. M., L. Polenzani, and R. Miledi. Characterization of bicuculline/baclofen-insensitive (ρ-like) γ-aminobutyric acid receptors expressed in *Xenopus* oocytes. II. Pharmacology of γ-aminobutyric acid$_A$ and γ-aminobutyric acid$_B$ receptor agonists and antagonists. *Mol. Pharmacol.* **43**:609–625 (1993).
36. Mihi, J. S., P. J. Whiting, R. L. Klein, K. A. Wafford, and R. A. Harris, The efficacy of benzodiazepine receptor ligands is determined by a single amino acid of the human GABA$_A$ receptor α2 subunit. *J. Biol. Chem.* **269**: 20380–20387 (1994).
37. Knoflach, F., K. H. Backus, T. Giller, P. Malherbe, P. Pflimin, H. Mohler, and G. Trube. Pharmacological and electrophysiological properties of recombinant GABAA receptors comprising the α3, β1 and γ2 subunits. *Eur. J. Neurosci.* **4**:1–9 (1992).
38. Pollard, S., M. J. Duggan, and F. A. Stephenson. Further evidence for the existence of a subunit heterogeneity within discrete γ-aminobutyric acid$_A$ receptor subpopulations. *J. Biol. Chem.* **268**:3753–3757 (1993).
39. Verdoorn, T. A. Formation of a heteromeric γ-aminobutyric type A receptor containing two different α subunits. *Mol. Pharmacol.* **45**:475–480 (1994).
40. McKernan, R. M., K. Quirk, R. Prince, P. A. Cox, N. P. Gillard, I. Ragan, and P. Whiting. GABA$_A$ receptor subtypes immunopurified from rat brain with α subunit-specific antibodies have unique pharmacological properties. *Neuron* **7**:667–676 (1991).
41. Duggan, M. J., S. Pollard, and F. A. Stephenson. Immunoaffinity purification of GABA$_A$ receptor α-subunit iso-oligomers: demonstration of receptor populations containing $\alpha_1\alpha_2$, $\alpha_1\alpha_3$ and $\alpha_2\alpha_3$ subunit pairs. *J. Biol. Chem.* **266**:24778–24784 (1991).
42. Laurie, D. J., P. H. Seeburg, and W. Wisden. The distribution of 13 GABA$_A$ receptor subunit mRNAs in the rat brain. II. Olfactory bulb and cerebellum. *J. Neurosci.* **12**:1063–1076 (1992).

Send reprint requests to: Povl Krogsgaard-Larsen, Department of Medicinal Chemistry, The Royal Danish School of Pharmacy, 2 Universitetsparken, DK-2100 Copenhagen, Denmark.