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Point mutations affecting antagonist affinity and agonist dependent gating of $GABA_A$ receptor channels

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Two variant amino acid sequences, which differ in a single amino acid residue, have been reported for the α1-subunit of the rat brain $GABA_A$ receptor. We separately co-expressed these two variants in *Xenopus* oocytes, in combination with β2 and γ2. This experiment showed that substitution of α1-Phe64 by Leu strongly decreases the apparent affinity for GABA dependent channel gating from 6 μM to 1260 μM. Starting from this observation, we used *in vitro* mutagenesis to obtain information relevant for the localization of the agonist/antagonist binding site in the $GABA_A$ receptor. Homologous mutation in α5 had similar consequences for α5β2γ2. Homologous mutation in β2 and γ2 resulted in intermediate and small shifts in EC_{50}, respectively. The apparent affinities of the competitive antagonists bicuculline methiodide and SR95531, the latter sharing close structural similarity with the agonist GABA, were decreased 60- to 200-fold by these mutations in α-subunits. Interestingly, these affinities remained nearly unaffected upon introduction of the homologous mutations in β2 and γ2, or upon mutation of the neighbouring amino acid in α1, Phe65 to Leu. These results suggest close functional and structural association of α-subunits with the agonist/antagonist binding site, and involvement of N-terminal portions of the extracellular domains of all subunits in the gating of the channel.

Key words: GABA/$GABA_A$ agonists/$GABA_A$ antagonists/$GABA_A$ receptor/ligand binding site

Introduction

The $GABA_A$ receptor is one of the sites of action of the major inhibitory neurotransmitter γ-aminobutyrate (GABA). An anion selective channel is an integral part of the $GABA_A$ receptor. Based on the biochemical isolation of a receptor protein from bovine cortex (Sigel *et al.*, 1983; Sigel and Barnard, 1984), cDNAs coding for two subunits have initially been cloned (Schofield *et al.*, 1987). These two subunits showed homology to subunits of the nicotinic acetylcholine and glycine receptors (Schofield *et al.*, 1987; Grenningloh *et al.*, 1987). Recently, molecular biological approaches have led to the identification of five families of GABA receptor subunit isoforms named α, β, γ, δ and ϱ with a total of 15 members up to the present (Schofield *et al.*, 1987; Levitan *et al.*, 1988; Khrestchatisky *et al.*, 1989; Pritchett and Seeburg, 1990; Luddens *et al.*, 1990; Ymer *et al.*, 1989a,b; Shivers *et al.*, 1989; Malherbe *et al.*, 1990a,b; Cutting *et al.*, 1991; Wilson-Shaw *et al.*, 1991). In analogy to nicotinic acetylcholine receptors the GABA channel is thought to be composed of five subunits surrounding the channel pore (for review see Olsen and Tobin, 1990). At present, it is not known which of the GABA receptor subunits collaborate to form a channel *in situ* in any cell type.

Little is known about the localization of agonist and competitive antagonist binding sites on the receptor complex. Specifically, it is not known which of the receptor subunits is responsible for GABA dependent channel gating. In the *Xenopus* oocyte, expression of a large number of subunit combinations suggested that at least one α-subunit and at least one β-subunit are required for the expression of large GABA gated currents (Sigel *et al.*, 1990). However, it appears that none of the subunits α, β or γ2 is absolutely required for the observation of GABA dependent channel gating (Sigel *et al.*, 1990). Either the γ2-subunit (Pritchett *et al.*, 1989; Sigel *et al.*, 1990) or the γ3-subunit (Knoflach *et al.*, 1991) seems to be required for benzodiazepine modulation of the GABA current. Expression of a combination of at least one α-, one β- and one γ-subunit is thus probably required to form a channel with properties observed *in situ*.

In the course of our previous work, we noticed that while the subunit combination α5β2γ2 reproduced faithfully generally accepted GABA channel properties, the subunit combination α1β2γ2 did not. In our study, the channel formed by α1β2γ2 showed peculiar gating characterized by an EC_{50} of ~1 mM, i.e. ~100 times higher than normal, and little cooperativity (Sigel *et al.*, 1990). This was surprising because these three subunits are extensively co-localized in the brain and the α1-subunit is the most abundant α-subunit (Séquier *et al.*, 1988; Malherbe *et al.*, 1990a). Review of the literature showed that the rat α1 used in our study differed by one amino acid from other published sequences for this subunit (Lolait *et al.*, 1989; Khrestchatisky *et al.*, 1989). In the sequence published by Lolait *et al.* (1989) and Khrestchatisky *et al.* (1989), position 64 is occupied by Phe whereas in the subunit previously isolated by us from a 7 day old rat brain cDNA library, this position is occupied by Leu (Malherbe *et al.*, 1990b).

We now show that this single amino acid change in the channel expressed from α1β2γ2 cRNAs in *Xenopus* oocytes has a profound influence on the gating by GABA and on the affinities of the competitive GABA antagonists bicuculline methiodide and SR95531 [2-(3-carboxypropyl)-3-amino-6-*p*-methoxyphenyl-pyridazinium bromide]. Furthermore, we have studied the effect of homologous point mutations in the subunits α5, β2 and γ2. Our data imply involvement in agonist gating of GABA channels of subunit portions located considerably closer to the N-terminal than previously suggested for the gating of nicotinic acetylcholine and glycine receptor channels. Furthermore, they suggest

importance of the amino acid at position 64 in the α1-subunit for the binding site(s) for GABA and competitive antagonists in recombinant $\alpha1\beta2\gamma2$ channels. Finally, our data imply that the properties of the $\alpha1\beta1\gamma2$ (Malherbe *et al.*, 1990c) and the $\alpha1\beta2\gamma2$ (Sigel *et al.*, 1990) channels previously described by our laboratories reflect the presence of the $\alpha1$ variant of a rare mutant animal.

Results

A point mutation affecting the response to GABA

An initial homology screening of 7 day old rat brain cDNA unamplified library in λgt11 with a bovine $\alpha1$ cDNA probe revealed six positive clones with various intensities. Two clones, OTRαF1 and OTRαF2, that gave strongest signals, were chosen for sequence analysis. The DNA sequence of clone OTRαF2 has previously been reported (Malherbe *et al.*, 1990b). DNA sequence analysis of clone OTRαF1 showed that it is identical to OTRαF2, except for a larger 5′-untranslated region. The sequence comparison of these overlapping clones with that of rat $\alpha1$ published by Lolait *et al.* (1989) and Khrestchatisky *et al.* (1989) revealed that the two cDNAs isolated by us contained an altered sequence having a T→C base change at position 306 (Malherbe *et al.*, 1990b). This base substitution results in the replacement of the conserved phenylalanine at position 64 with a leucine in the α1-subunit (Figure 1). In order to rule out that this mutation had been introduced during the cDNA cloning steps, a polymerase chain reaction (PCR) product was generated from the poly(A)$^+$ RNA of 7 day old rat brain (the same mRNA material already used for making the cDNA λgt11 library) with a sense and antisense primer pair flanking the region of the base alteration of rat $\alpha1$. The resulting PCR product was cloned into M13mp18 and the single-stranded DNAs from 10 individual plaques were sequenced. A change of base T→C at position 306 was observed in all the sequences analysed (data not shown). Therefore, the isolated poly(A)$^+$ RNA probably stems from mutated neonatal rat brains. This assumption was further substantiated by additional experiments. PCR-mediated DNA amplification was performed with cDNAs from individual rat brains (2 days old, 7 days old and adult). The PCR products were subcloned into M13 vector. In all the 35 phages (10 from 2 day old, 14 from 7 day old and 11 from adult) sequenced, the base T, not C as in the mutant, was found to be present at position 306.

To compare the properties of our mutated $\alpha1^{64Leu}$ clone with that of $\alpha1^{64Phe}$ (Lolait *et al.*, 1989; Khrestchatisky *et al.*, 1989), the nucleotide C306 in the clone OTRαF2 was changed to T306 by site-directed mutagenesis. Both variants, $\alpha1^{64Phe}$ and $\alpha1^{64Leu}$, were co-expressed individually in combination with $\beta2$ and $\gamma2$ in *Xenopus* oocytes. Both triple combinations resulted in the efficient formation of channels as indicated by the expression of average maximal GABA current amplitudes >7 μA. Figure 2A and B show typical current traces in response to different concentrations of GABA, in oocytes injected with $\alpha1^{64Phe}\beta2\gamma2$ and $\alpha1^{64Leu}\beta2\gamma2$, respectively. It is evident that the mutation strongly affects the sensitivity of the channels to the agonist GABA. These two agonist response curves were collected in two different batches of oocytes. As we observed some variation (maximally ~4-fold) in the EC_{50} for GABA between different batches of oocytes expressing the same

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α1 64Phe          SFGPVSDHDMEYTIDVFFRQSWKDERL 74
                                64
α1 64Leu          SFGPVSDHDMEYTIDVLFRQSWKDERL
                                  65
α1 64Phe;65Phe-Leu SFGPVSDHDMEYTIDVFLRQSWKDERL

α5                SFGPVSDTEMEYTIDVFFRQSWKDERL 78
                                68
α5 68Phe-Leu      SFGPVSDTEMEYTIDVLFRQSWKDERL

β2                SIDMVSEVNMDYTLTMYFQQAWRDKRL 72
                                62
β2 62Tyr-Leu      SIDMVSEVNMDYTLTMLFQQAWRDKRL

γ2                SIGPVNAINMEYTIDIFFAQTWYDRRL 87
                                77
γ2 77Phe-Leu      SIGPVNAINMEYTIDILFAQTWYDRRL
                  *   *    * **      * * * **
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Fig. 1. Comparison of the region immediately surrounding the mutation site (64Leu) in the rat $GABA_A$ receptor α1-subunit sequence with the corresponding sequences of rat α5-, β2- and γ2-subunits. The mutated amino acids are shown by bold letters. The numbering of amino acids corresponds to the mature proteins. *indicates match in all sequences.

subunit combination, we directly compared the two subunit combinations in the same batches of oocytes. Figure 2C shows averaged data from two series of such agonist response experiments. It is evident that substituting Phe in position 64 by Leu leads to a drastic, ~200-fold, shift in the agonist sensitivity towards higher concentrations. Table I summarizes averaged EC_{50} values derived from fits to the GABA response curves. Coinciding with the shift we observed a significant decrease in the cooperativity of GABA gating, as obtained from log-log plots (Table I). This is not obvious from the averaged data in Figure 2C, but became clear from log-log plots of individual dose–response curves. The latter analysis is restricted to data points obtained at low agonist concentration.

We also analysed for further differences caused by the point mutation. The reversal potential of the current was not significantly affected by the mutation. Often, the rise time to peak current was observed to be faster for oocytes expressing $\alpha1^{64Phe}\beta2\gamma2$ than in oocytes expressing $\alpha1^{64Leu}\beta2\gamma2$. However, this time course could not be properly resolved with the present techniques used. The time course of current decay in the presence of saturating concentrations of GABA was also compared. Desensitization of the current expressed from both combinations was satisfactorily described by a double exponential decay and a residual, time-independent current component. Only the time-independent component was significantly ($P < 0.005$) affected (not shown). Substitution of Phe64 by Leu increased this current fraction from 0.046 ± 0.011 (n = 10) to 0.065 ± 0.011 (n = 11).

Altered sensitivity to competitive antagonists

From the EC_{50} data for GABA dependent channel gating it is not clear whether the Phe64 to Leu substitution affects the affinity of GABA to bind to the channel protein or the subsequent conformational transitions of the protein that ultimately lead to the opening of the channel pore. Therefore, we analysed the currents expressed by the two subunit combinations $\alpha1^{64Phe}\beta2\gamma2$ and $\alpha1^{64Leu}\beta2\gamma2$ for their sensitivities to the competitive GABA antagonists bicuculline

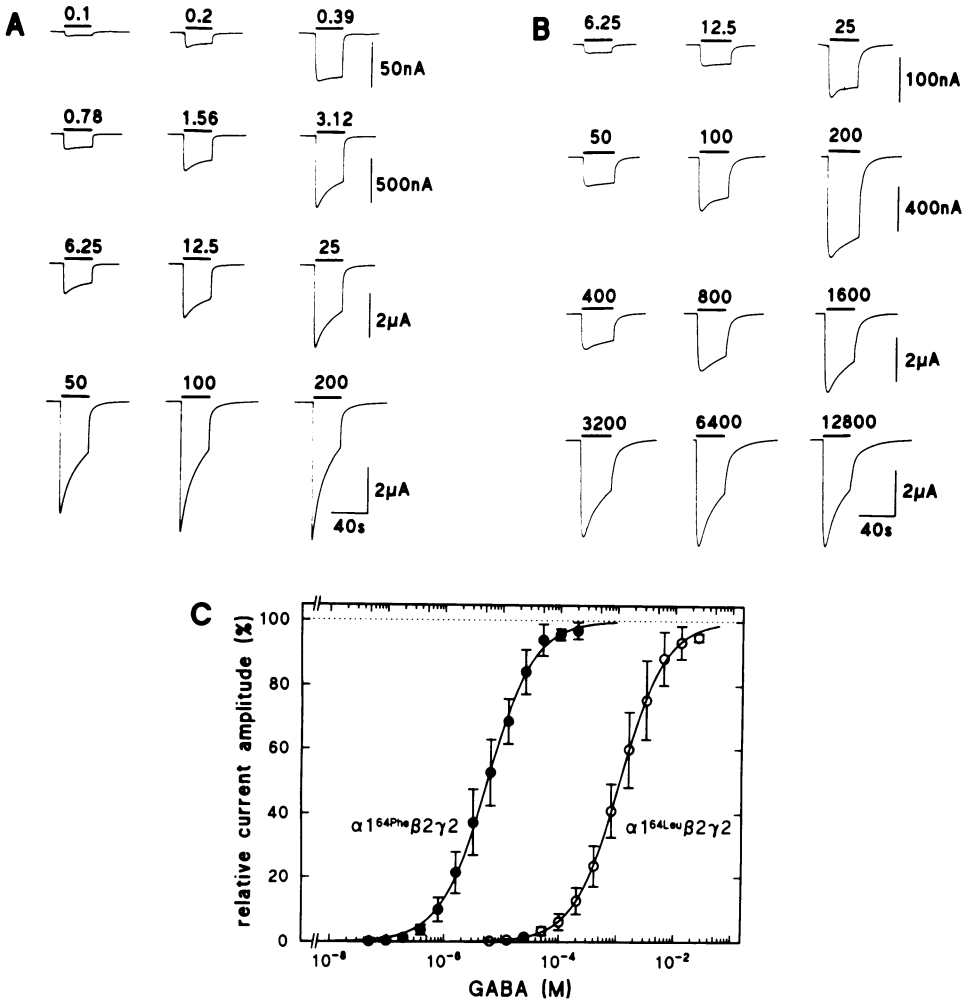

Fig. 2. Expression of $\alpha1^{64Phe}\beta2\gamma2$ and $\alpha1^{64Leu}\beta2\gamma2$. Agonist response curves. (**A**) Current traces obtained from an oocyte expressing $\alpha1^{64Phe}\beta2\gamma2$. (**B**) Current traces obtained from an oocyte expressing $\alpha1^{64Leu}\beta2\gamma2$. The bars indicate the time period of GABA perfusion; the numbers indicate the GABA concentration in μM. (**C**) Agonist response curves: ●, $\alpha1^{64Phe}\beta2\gamma2$ and ○, $\alpha1^{64Leu}\beta2\gamma2$. Six dose–response curves obtained from oocytes originating from two different donor animals were individually fitted as indicated in Materials and methods, and standardized to I_{max} = 100%. Standardized current amplitudes are shown as average and SD. Data were refitted to give the solid curve.

methiodide and SR95531. For these inhibition experiments we used a GABA concentration eliciting ~10% of the maximal current amplitudes. Figure 3 shows that there is a drastic, ~200-fold loss of sensitivity to bicuculline methiodide as a consequence of the replacement of Phe64 by Leu. This indicates that the binding site of bicuculline methiodide is affected by the single amino acid change. A similar change in the apparent affinity was observed for SR95531 (Table I).

Effect of the homologous substitution in α5

We were interested to analyse a homologous mutation in another α-subunit. For this purpose, Phe68 in α5, homologous to Phe64 in α1, was replaced by Leu (Figure 1). Table I summarizes the data on the functional properties of the mutated subunit after combined expression with β2γ2. The mutation in α5 caused a slightly smaller shift in both the apparent affinity for GABA gating and the sensitivity to bicuculline methiodide than the mutation in α1. However, there was a similar decrease in the cooperativity of GABA gating. GABA current amplitudes were similar to those with wild type α5, indicating that the mutation did not interfere with efficient biosynthesis.

Effect of homologous mutations in β2 and γ2

Analogous mutations in the β2- and γ2-subunits were also investigated (Figure 1). The change to Leu of Tyr62 of β2 and Phe77 of γ2 also resulted in both cases in a change of the apparent affinity of GABA gating when co-expressed with $\alpha1^{64Phe}$ and either γ2 or β2 ($\alpha1^{64Phe}\beta2^{62Tyr\text{-}Leu}\gamma2$ and $\alpha1^{64Phe}\beta2\gamma2^{77Phe\text{-}Leu}$, Table I). For the mutation in the β2-subunit the shift was ~30-fold as compared with a 200-fold shift by the mutation in the α1-subunit. The mutation in the γ2-subunit caused only about a 6-fold shift. Thus, point mutations not only in the α-subunits but also in the β- and γ-subunits affect the EC_{50} for GABA, though to different extents. Interestingly, alterations of β2- and of γ2-subunits did not significantly affect the apparent affinities for the competitive GABA antagonists bicuculline methiodide and SR95531, nor the Hill coefficient of GABA gating (Table I). Mutation of the γ2-subunit did not decrease the maximal current amplitude expressed, while mutation of the β2-subunit led to expression of ~5-fold smaller current amplitudes than the non-mutated control subunit combination, indicating that the mutation in β2, but not in γ2 might somewhat interfere with efficient assembly of functional channels.

Table I. Properties of mutant $GABA_A$ receptor channels

Subunit combination	EC_{50} (μM; GABA)	n	IC_{50} (μM; bicuculline methiodide)	IC_{50} (μM; SR95531)
$\alpha1^{64Phe}\beta2\gamma2$	6 ± 2	1.6 ± 0.1	0.9 ± 0.3	0.10 ± 0.02
$\alpha1^{64Leu}\beta2\gamma2$	1260 ± 380[a]	1.3 ± 0.1	200 ± 65	5.7 ± 1.5
$\alpha1^{64Phe}\beta2^{62Tyr\text{-}Leu}\gamma2$	181 ± 30[b]	1.6 ± 0.1	0.9 ± 0.1	0.07 ± 0.01
$\alpha1^{64Phe}\beta2\gamma2^{77Phe\text{-}Leu}$	33 ± 15	1.6 ± 0.0	0.9 ± 0.1	0.08 ± 0.03
$\alpha1^{64Phe;65Phe\text{-}Leu}\beta2\gamma2$	183 ± 47	1.6 ± 0.2	1.8 ± 1.0	0.17 ± 0.04
$\alpha5\beta2\gamma2$	11 ± 4	1.7 ± 0.1	0.6 ± 0.1	–
$\alpha5^{68Phe\text{-}Leu}\beta2\gamma2$	320 ± 100[c]	1.3 ± 0.1	53 ± 19	–

EC_{50} and Hill coefficients (n) for GABA currents and IC_{50} for bicuculline methiodide inhibition of GABA currents were determined as shown in Materials and methods. The inhibition of currents by bicuculline methiodide and SR95531 were determined at a GABA concentration eliciting ~10% of the maximal response. Each value indicates the mean ± SD of at least six experiments performed with different oocytes from two different donor animals.

[a] A slightly better fit was obtained by assuming two components with EC_{50} 450 μM and 1300 μM, the first component accounting for 37% of I_{max}.

[b] A slightly better fit was obtained by assuming two components with EC_{50} 120 μM and 1350 μM, the first component accounting for 80% of I_{max}.

[c] A slightly better fit was obtained by assuming two components with EC_{50} 40 μM and 540 μM, the first component accounting for 21% of I_{max}.

–, not analysed.

Specificity of the α1 Phe64 to Leu substitution

In order to assess the specificity of the Phe64 to Leu substitution we exchanged the neighbouring amino acid in subunit $\alpha1$, Phe65, for Leu (Figure 1), and tested for GABA, bicuculline methiodide and SR95531 sensitivity. This change also decreased the apparent affinity for GABA dependent gating, though to an ~7-fold smaller extent than the change in Phe64. The decrease of the sensitivities for bicuculline methiodide and SR95531 was only very small, ~2-fold as compared with 220- and 60-fold for the change in the neighbouring amino acid (Table I). Thus, individual alterations in one of the two neighbouring Phe residues to Leu have drastically different consequences.

Mutations in different, more distant areas were also investigated for their effect on EC_{50} for GABA and IC_{50} for bicuculline methiodide. Human $\alpha1$ corresponds to rat $\alpha1^{64Phe}$, with a point mutation at position 94, Trp→Arg, and a Leu inserted after residue 3. Interestingly, Trp94 is homologous to Trp86 of the *Torpedo* nicotinic acetylcholine receptor α-subunit, which is labelled in photoaffinity labelling experiments using a competitive antagonist probe (Galzi *et al.*, 1991). Co-expression of human $\alpha1$ with rat $\beta2\gamma2$ led to channels with an EC_{50} for GABA of 28 ± 7 μM (n = 4), and with an IC_{50} for bicuculline methiodide of 1.6 ± 0.3 μM (n = 3, both single batch of oocytes). As mentioned above, the EC_{50} for GABA was somewhat variable between different batches of oocytes. In this batch of oocytes $\alpha^{64Phe}\beta2\gamma2$ resulted in currents characterized by an EC_{50} of 25 ± 5 μM (n = 4). Thus, these two non-conservative changes in the N-terminal domain, but positions distant from Phe64 have only very little effect on GABA dependent channel gating, and on the IC_{50} for bicuculline methiodide.

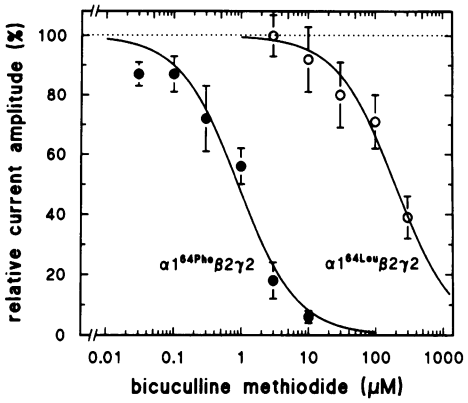

Fig. 3. Concentration dependent inhibition of GABA currents by bicuculline methiodide: ●, $\alpha1^{64Phe}\beta2\gamma2$ and ○, $\alpha1^{64Leu}\beta2\gamma2$. GABA was applied at a concentration eliciting ~10% of the respective maximal current response, alone or in combination with different concentrations of bicuculline methiodide. The curves were fitted with the equation $I(c) = I_0/(1 + c/IC_{50})$, where c is the concentration of bicuculline methiodide and I_0 (= 100%), the control current amplitude in the absence of inhibitor.

Discussion

We have investigated the effect of conservative hydrophobic amino acid substitutions in the N-terminal domain of GABA receptor subunits. Mutation of the Phe64→Leu in the α-, but not of homologous mutation in the $\beta2$- and $\gamma2$-subunits, has a profound impact on the apparent affinity of the competitive GABA antagonists bicuculline methiodide and SR95531 for the $\alpha1\beta2\gamma2$ channel expressed in *Xenopus* oocytes. Similarly, the EC_{50} for GABA is strongly affected by alteration of the α-, and, only to a much lesser extent, of the $\beta2$- and $\gamma2$-subunits. The effects of this mutation are specific as substitution of Phe65 by Leu resulted in much smaller if any effects on these parameters.

Alteration of the properties of the channel formed by a combination containing a mutated subunit could, in principle, be due to loss of ability of the latter to assemble. This is clearly not the case, as formation of functional GABA channels expressed from a single type of α-, in combination with $\gamma2$-subunits has not been observed in the oocyte expression system. In addition, expression of $\beta2\gamma2$ only results in inefficient expression (Sigel *et al.*, 1990). Expression of $\alpha1\beta2\gamma2$ containing a point mutation in one of the subunits resulted, as shown here, in the expression of large current amplitudes.

The size of the effect of the Phe to Leu substitution in amino acid residue 64 of $\alpha1$ is surprising, if one considers that both amino acids are hydrophobic and that Leu occupies a significantly smaller volume than Phe (Chotia, 1975). The 220-fold change in binding affinity for bicuculline methiodide

amounts to a 3.3 kcal/mol change in standard free energy of binding. This corresponds approximately to the loss of one hydrogen bond in the receptor–antagonist complex upon replacement of Phe by Leu in position 64 of the α1-subunit.

$\alpha 1^{64Phe}\beta 2\gamma 2$ forms a channel with normal gating properties

mRNA coding for α1 is the most abundant α-subunit mRNA expressed in rat brain (Séquier *et al.*, 1988; Malherbe *et al.*, 1990a). On the protein level, it has recently been shown that subunit specific antibodies directed against the rat α1-subunit immunprecipitate ~40% of the benzodiazepine antagonist binding sites, more than antibodies directed against the α2-, α3- and α5-subunits (McKernan *et al.*, 1991). Although the latter finding should be interpreted with care because only binding sites soluble in 0.5% deoxycholate are considered, the finding underlines the quantitative importance of α1. We have previously stated that expression of $\alpha 1\beta 2\gamma 2$ in *Xenopus* oocytes results in channels with properties very different from those seen after expression of total brain mRNA (Sigel *et al.*, 1990), which are those generally accepted for natural GABA channels. In the light of our present data this is not correct as long as position 64 of α1 is occupied by Phe. In this case, α1-containing channels display cooperative GABA dependent gating with normal EC_{50}.

Functional agonist and antagonist binding sites in $\alpha 1\beta 2\gamma 2$

From a study of many different subunit combinations expressed in the *Xenopus* oocyte (Sigel *et al.*, 1990) it is evident that none of the α-, β- or γ2-subunits is absolutely required for the formation of GABA gated channels. Large currents are expressed from various αβ subunit combinations and at least some current is seen after expression of $\beta 2\gamma 2$ and of $\alpha 3\alpha 5\gamma 2$. It is generally assumed that two GABA binding sites cooperate in the opening of a channel, but it is not known where the functional binding sites for the agonist GABA are located, neither in a channel composed of α1, β2 and γ2-subunits, nor in any other GABA activated channel.

Bicuculline methiodide and SR95531 behave in binding assays and in electrophysiological experiments as competitive antagonists of GABA (for review see Wermuth and Bizière, 1986). The most simple interpretation of a competitive antagonist is that it shares at least part of the binding pocket with the agonist, in such a way that occupancy of the site is mutually exclusive. This interpretation is not unlikely in view of the fact that at least the competitive antagonist SR95531 has a close structural similarity to the agonist GABA. Effects of mutations on the apparent affinity of GABA gating are either due to alteration of initial binding of GABA, or due to an influence on the subsequent conformational changes that lead to the opening of the channel, whereas effects on the apparent affinity of the antagonist would reflect alteration at the GABA binding pocket.

If this simple model of competitive antagonism holds true for the GABA receptor, our results are compatible with the notion that the α-subunits carry an important part of the agonist/antagonist binding site. In line with such a suggestion is the fact that cooperativity of gating is only affected by the mutations in the α-subunits.

However, it should be noted here, that our results do not necessarily imply a direct involvement of residue 64 of subunit α1 in the binding of GABA, bicuculline methiodide or SR95531. Replacement of the aromatic amino acid Phe by Leu may also lead to a larger modification in the structure of the extracellular domain of the α1-subunit, which would then exert a strong influence on the binding sites for bicuculline methiodide and SR95531.

Interaction between α1-, β2- and γ2-subunits

It is clear from our data that the conformation of the extracellular domain of any of the subunits α1, β2 and γ2 is important for the gating of the channel formed by these subunits. Based on our findings we favour an interpretation that postulates (i) a conformational change in the extracellular domain caused by the above single point mutations in any of the mutated subunits, and (ii) an intersubunit transfer of this conformational change to other subunits of the channel complex, resulting in an altered apparent affinity for channel gating by GABA. This intersubunit transfer has little, intermediate and strong impact on GABA dependent channel gating if it originates from the γ2-, β2- and α1-subunits, respectively. The fact that only the amino acid substitution in the α-subunits, but not in the β2- and γ2-subunits affects the apparent cooperativity points to a crucial role for the α-subunits in cooperativity of agonist dependent channel gating.

Other determinants of $GABA_A$ channel gating

Different structures are known to affect the apparent affinity for GABA gating. If position 64 of the α1-subunit is occupied by Phe we observe high, and if occupied by Leu low apparent affinity of gating. In the α3-subunit a Phe is located in the homologous position of α1-Phe64 and, in spite of this, the subunit confers low affinity gating to the channel expressed in combination with β2 and γ2 in *Xenopus* oocytes (Sigel *et al.*, 1990). In the ρ-subunit, which efficiently produces homomeric channels with high affinity gating after expression in the *Xenopus* oocyte, tyrosine occupies the homologous position of α1-Phe64 (Cutting *et al.*, 1991). In the *Xenopus* oocyte expression system, it has been shown that additional co-expression of γ2 with a given subunit combination leads to an increase in the EC_{50} for GABA (Sigel *et al.*, 1990).

β-subunits have previously been implicated in GABA binding (Casalotti *et al.*, 1986; Browning *et al.*, 1990). This proposal is based on the finding that in photoaffinity labelling experiments the agonist [^{3}H]muscimol labels subunits migrating with an apparent molecular weight higher than that of a subunit labelled with benzodiazepines (Casalotti *et al.*, 1986), and which is recognized by an anti-α1 antibody (Browning *et al.*, 1990). This finding should be considered with some reservation, as the relationship of these higher molecular weight components to individual cloned subunits is not yet clarified (Browning *et al.*, 1990). Furthermore, in such experiments polypeptide regions quite distant from the labelled ligand may react and become labelled. Also, binding studies are more likely to reveal agonist binding to the desensitized form of an ion channel.

Putative agonist binding domains in other ligand gated channels

The point mutation described here for the α1-subunit is located at 161 amino acid residues distance from the first putative transmembrane domain M1. Amino acid residues located closer to M1 have been implicated in the gating of the glycine and the nicotinic acetylcholine channel.

Replacement of Glu167 by Gly in the glycine receptor subunit α2* resulted in a functional channel with agonist and antagonist response curves shifted to higher concentrations (Kuhse *et al.*, 1990). This point mutation is located 59 amino acids distant from M1. In the nicotinic acetylcholine channel the region involved in the interaction with both the agonist nicotine (Middleton and Cohen, 1991) and competitive antagonists (for review see Galzi *et al.*, 1991) have been mapped with photoaffinity labelling on the α-subunit of the *Torpedo* receptor. Amino acids at a distance of 18–21 amino acid residues from M1 were radioactively labelled in these experiments. Point mutations in the same region have been shown to profoundly affect agonist affinity (Tomaselli *et al.*, 1991). Antagonists additionally labelled amino acids at a distance of 62 and 118 amino acids from M1. The most N-terminally located amino acid labelled is homologous to residue 124 in the rat GABA receptor α1-subunit. Thus, the present work provides evidence for involvement of amino acid portions relatively close to the N-terminus within subunit α1 in the response of the GABA channel to its agonist.

From elegant work by Lo *et al.* (1990, 1991) it is evident that not only the extracellular part of ligand gated ion channels is important for the gating process. Thus, it has been shown that subtle changes in the amino acid residues in the first putative transmembrane domain M1 of the γ-subunit of the nicotinic acetylcholine receptor strongly affect open–close transitions of the channel. Recently, it has also been shown that in chick brain homo-oligomeric acetylcholine receptor point mutations of Leu246, putatively lining the ion conducting pore of the channel, lead to drastic changes in the apparent affinity for acetylcholine for channel gating (Revah *et al.*, 1991).

Physiological significance of the α1 Phe to Leu mutation

In view of the large decrease in the GABA sensitivity caused by the α1 Phe→Leu mutation, it is surprising that the mutant rat survived until day 7 after birth. In the adult rat α1 is the predominant α-subunit (Behnke *et al.*, 1991; Duggan *et al.*, 1991; McKernan *et al.*, 1991). However, expression of α1 occurs relatively late in development, reaching a maximum only at about days 14–21 after birth, depending on the brain area (Gambarana *et al.*, 1990). Other subunits could have fulfilled a life sustaining role in the young mutant animal. Alternatively, in the young animal α1 may not necessarily associate with β2γ2 to form a channel as is thought to be the case in adult animals (Behnke *et al.*, 1991; Gambarana *et al.*, 1991). A third possibility is that our measurements, which are restricted to phenomena occurring later than ~0.5 s after addition of the agonist, do not properly reflect phenomena important for synaptic GABAergic transmission. Faster initial responses could be more significant. However, in the absence of relevant data it is difficult to speculate about the physiology of the mutant animal. Clearly, transgenic animals are now needed for such an analysis.

Conclusions

Our observations shed light on the complex question of where the functional agonist binding in a recombinant GABA channel takes place and how the subunits interact to enter the open channel conformation. A precise evaluation of the structural changes induced by the mutation studied here has to await determination of the structure of the extracellular domains of ligand gated receptor subunits.

Materials and methods

The original library containing $\alpha1^{64Leu}$ was obtained from three male, albino, random outbred rats of Wistar origin from the same litter. The animals were obtained from Biological Research Laboratories, Füllinsdorf, Switzerland. The control animals for PCR analysis, which led to identification of $\alpha1^{64Phe}$, were obtained 3 years later from the same source.

Site-directed mutagenesis

Oligonucleotide-directed site specific mutagenesis was performed using a kit supplied by Amersham (UK), under the conditions recommended by the supplier. For site-directed mutagenesis, the following oligonucleotides (synthesized by Genosys Biotechnologies Inc., Texas) were used:
5′-GCTTTGGCGGAAAAACACATCTATTGTAT-3′ (α1 64Leu→Phe);
5′-CTTCCAGCTTTGGCGGAGAAACACATCTATTGTAT-3′
(α1 65Phe→Leu);
5′-AGCTTTGACGGAAAAGTACATCTATGGTATA-3′
(α5 68Phe→Leu);
5′-GACTACACCTTGACCATGCTTTTCCAGCAAGCCTGGAG-3′
(β2 62Tyr→Leu);
5′-GAATACACAATTGATATTCTTTTTGCCCAAACCTGG-3′
(γ2 77Phe→Leu).
All the mutations were created in M13mp18 carrying the cDNA for $GABA_A$ receptor subunits. Single-stranded DNA of the recombinant phage was used as starting material for the mutagenesis of the subunit gene. The complete DNA inserts of all the mutants were sequenced to ensure that the correct mutations were introduced without unwanted side mutations. The SP6 plasmids containing the mutated genes were constructed by subcloning the *Eco*RI insert from M13 into pSPT19 plasmid (Pharmacia).

Expression of cloned subunit isoform combinations in Xenopus oocytes

The *in vitro* transcription, capping and polyadenylation of the rat brain subunit isoforms have been described elsewhere (Malherbe *et al.*, 1990a,b,c). The cRNA combinations were co-precipitated in ethanol, shipped and stored below 0°C. Isolation of follicles from the frogs, culturing of the follicles, injection with RNA, and removal of the follicular cell layers from the oocytes were all performed as described earlier (Sigel, 1987). Follicles were injected with ~50 nl of the capped transcripts. This solution contained the transcripts coding for each of the different subunits at a concentration of 50–100 nM, to allow injection of stoichiometric amounts. The follicular cell layers were removed from the oocytes 1 or 2 days prior to the electrophysiological experiments.

Electrophysiological experiments

All electrophysiological measurements were carried out on denuded oocytes. Oocytes were placed in a 0.4 ml bath on a nylon grid, and the bath was perfused throughout the experiment at 6 ml/min with 90 mM NaCl/1 mM KCl/1 mM $MgCl_2$/1 mM $CaCl_2$/5 mM HEPES–NaOH (pH 7.4). The perfusion medium could be switched to one supplied with GABA alone or in combination with either bicuculline methiodide or SR95531. All experiments were carried out at room temperature (23–29°C). For the current measurements, oocytes were impaled with two microelectrodes and the membrane potential voltage-clamped at −80 mV.

Data on the gating properties of the different GABA channels expressed were obtained as follows (Sigel and Baur, 1988; Sigel *et al.*, 1990). Individual GABA dose–response curves obtained from GABA applications over a wide range of concentrations were fitted using a non-linear least-squares method (Gauss–Newton–Marquardt). The equation used was $I(c) = I_{max}\, c^n/(c^n + EC_{50}{}^n)$, where c is the GABA concentration in the perfusion medium, I the current elicited, EC_{50} the GABA concentration eliciting half maximal current (½ I_{max}) and n the Hill coefficient. Table I shows the average and SD of such data. Hill coefficients compiled in Table I were obtained from the limiting slope of the log-log plots of the dose–response curves obtained with low GABA concentrations. Therefore, it represents the Hill coefficient of the components exhibiting highest affinity for GABA, in cases where there is a heterogeneity. The same oocyte was exposed to increasing concentrations of GABA. Between two applications the oocyte was perfused with standard medium for 3–15 min (depending on the GABA concentration previously used) to ensure full recovery from desensitization.

Precautions were taken to minimize the effect of desensitization when current amplitudes were determined. For the measurement of dose–response curves over a wide GABA concentration range, the perfusion solution was applied through a glass capillary with an inner diameter of 1.35 mm, the mouth of which was placed ~0.4 mm from the surface of the oocyte. This allowed fast changes in agonist concentration around the oocyte. The extent of change was 70% in <0.5 s, as determined by switching the perfusion medium to a sodium-free medium, and measuring the amplitude of sodium currents expressed in oocytes injected with mRNA isolated from chick forebrain. Current amplitudes were always back-extrapolated to the time when the current had reached 50% of the maximal amplitude. Correction was usually very small, and amounted to 12% of the peak current amplitude in the worst case.

In control experiments, we verified in the following way that bicuculline methiodide and SR95531 are acting indeed in a competitive fashion on the recombinant channels expressed in the oocyte. GABA was used at a concentration eliciting 10% of the maximal current amplitude to establish concentration dependent inhibition by bicuculline methiodide and SR95531. The concentration of bicuculline methiodide or SR95531 producing 80% inhibition in these experiments was then tested for inhibition of the current produced by a 100-fold higher concentration of GABA. In such experiments very little inhibition by either bicuculline methiodide or SR95531 could be observed in all of the subunit combinations tested ($\alpha1^{64Phe}\beta2\gamma2$, $\alpha1^{64Leu}\beta2\gamma2$, $\alpha1^{64Phe}\beta2^{62Tyr\text{-}Leu}\gamma2$, $\alpha1^{64Phe}\beta2\gamma2^{77Phe\text{-}Leu}$, $\alpha1^{64Phe:65Phe\text{-}Leu}\beta2\gamma2$).

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