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MOLECULAR BIOLOGY OF THE $GABA_A$ RECEPTOR: FUNCTIONAL DOMAINS IMPLICATED BY MUTATIONAL ANALYSIS

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1. ABSTRACT

$GABA_A$ receptors are the major inhibitory neurotransmitter receptors in mammalian brain. They belong to a family of ligand-gated ion channels that also includes the nicotinic acetylcholine receptors, glycine receptors and $5HT_3$ receptors. Each receptor in the family is believed to be a pentamer of homologous subunits that assemble to form a central transmembrane ion pore which, in the case of the $GABA_A$ receptor, is anion-selective. For almost twenty years, there has been tremendous interest in the structure and function of $GABA_A$ receptors, not only because of their importance in regulating brain excitability but also because these proteins are the specific targets for a wide variety of therapeutic agents including the anxiolytic benzodiazepines and barbiturates. Molecular cloning has revealed that $GABA_A$ receptors are heterogeneous, being formed by combinations of different isoforms of several subunit classes (α, β, γ, δ). The physiological and pharmacological properties of individual $GABA_A$ receptor subtypes appear to depend on their precise subunit complement. In this review, we focus on the application of modern techniques in molecular biology, particularly mutational analysis, to identify structural domains of these receptors that are important for ligand recognition and receptor function.

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2. INTRODUCTION

γ-Aminobutyric acid (GABA) is the most abundant inhibitory neurotransmitter in the vertebrate central nervous system where it activates two major receptor types, namely $GABA_A$ and $GABA_B$ receptors. The former is a member of a ligand-gated ion channel superfamily (1) whereas the latter is believed to be a G-protein-coupled receptor, although this has

yet to be confirmed by cloning and expression studies. The $GABA_A$ receptor contains an integral ion channel which opens, as a consequence of GABA binding, to allow the flux of Cl^- ions. In most brain regions Cl^- ions pass down a concentration gradient into the cell to cause hyperpolarisation; however, depolarizing responses have been reported in specific neuronal populations (2) and at certain stages in development (3). $GABA_A$ and $GABA_B$ receptors are most readily distinguished by the actions of bicuculline and baclofen, the former being a $GABA_A$ receptor-specific antagonist while the latter is a $GABA_B$ receptor-specific agonist (4). Other receptor-selective agents exist, one of the most widely used of which is muscimol, a specific agonist at $GABA_A$ receptors (5).

The $GABA_A$ receptor has a very rich pharmacology and this has provided the impetus both for the identification of more (or better) therapeutic agents with which to modulate receptor function and also for the use of this protein as a model system to study complex interactions between multiple binding sites. A large number of modulators, that apparently bind to discrete sites on the $GABA_A$ receptor, have been identified. These include benzodiazepines, barbiturates, neurosteroids, some anesthetics, Zn^{2+}, avermectin and possibly also alcohols (reviewed in 6). Occupancy of one site can alter the characteristics of one or more of the others, including allosteric modulation of the binding of GABA and/or channel gating.

From a therapeutic perspective, the benzodiazepine site has proved to be the most important. Remarkably, it was some 15 years after the introduction of benzodiazepines into clinical practice that their site of action (*i.e.* the $GABA_A$ receptor) was elucidated (reviewed in 6). While benzodiazepines have both muscle relaxant and anticonvulsant properties, they have proved to be most useful in the treatment of anxiety and insomnia. The benzodiazepines that are used therapeutically are known as "agonists". These drugs potentiate the actions of GABA by causing a shift in the GABA concentration-effect curve to lower concentrations and increasing the frequency of channel opening without affecting either the channel open time or conductance (7).

The discovery of a number of structurally unrelated compounds that appeared to recognize the benzodiazepine site led to the intriguing observation that drugs that act at this site can elicit a spectrum of pharmacological activities ranging from full agonist through antagonists to inverse agonists (8). The latter group interacts with the receptor to produce an effect that is opposite to that of the classical agonists *i.e.* a rightward shift in the GABA concentration-effect curve and a decrease in the frequency of channel opening. Benzodiazepine antagonists, such as Ro15-1788 (more recently known as flumazenil), compete for the same binding site and thus block the effects of both agonists and inverse agonists, although alone they have no overt effects on GABA responses. A number of compounds display either agonist or inverse agonists properties but are less efficacious than others; consequently these agents have been termed partial agonists and partial inverse agonists, respectively. As discussed below, there has been intense interest in the elucidation of the structure-function relationships that determine the pharmacological profiles of the benzodiazepines and related compounds. In this regard, two benzodiazepines that have proved to be particularly useful are the agonist, flunitrazepam, and the partial inverse agonist, Ro15-4513. Both of these drugs are available in radiolabelled form and both can be used as photoaffinity reagents to probe the environment of the benzodiazepine binding site (for example, see 9).

The first indication of the inherent complexity of the $GABA_A$ receptor came from studies of drugs that compete for the benzodiazepine site. CL218872, a triazolopyridazine, was shown to have a differential ability to displace classical benzodiazepine agonists from their binding sites in different regions of the brain (10). This compound appeared to have a higher affinity for sites in rat cerebellum than for those in the hippocampus. Thus, in these early studies, two types of benzodiazepine sites, designated BZI and BZII respectively, were pharmacologically distinguished. This provided the first evidence, later to be confirmed by molecular cloning, that $GABA_A$ receptors are heterogeneous.

Knowledge that the $GABA_A$ receptor carried a high affinity binding sites for benzodiazepines, spurred attempts to purify the receptor by benzodiazepine affinity chromatography. Early purification studies and biochemical characterization suggested that the $GABA_A$ receptor was formed by an α and a β subunit which could be photoaffinity labelled, respectively, by [^{3}H]flunitrazepam (11) and by [^{3}H]muscimol (12, 13). In 1987, the results of a fruitful collaboration between the groups of Eric Barnard and Peter Seeburg provided the first $GABA_A$ receptor cDNA sequences (14). Sequences of an α and β subunit were obtained by a cloning strategy which was based on partial amino acid sequences of the purified receptor subunits. In the same issue of Nature, the sequence of a glycine receptor (GlyR) subunit was reported (15). Comparison of these cDNA sequences led to the landmark discovery that, not only were the sequences of the two $GABA_AR$ subunits and the GlyR subunit related to each other but they were also homologous to the previously reported sequences of the four subunits of the *Torpedo* nicotinic acetylcholine receptor (nAChR; 16). Thus, for the first time, it was clear that these proteins belonged to a structurally related "superfamily" of ligand-gated ion channels (LGIC). More recently, another member of this family has been identified *i.e.* the 5-hydroxytryptamine type 3 ($5HT_3$) receptor (17). As discussed below, cloning studies have since demonstrated the existence of a large family of $GABA_A$ receptor subunit genes and, like many other CNS receptors in the LGIC superfamily, the potential for receptor heterogeneity is much greater than

had earlier been anticipated from pharmacological characterization.

Imaging of the nAChR (see 18, 19) and, more recently, of the $GABA_A$ receptor (20) by electron microscopy has suggested that each member of the LGIC family is a pentamer of subunits that are assembled around a central ion channel. The mature subunits of all members of the superfamily are predicted to have a similar overall structure. Each subunit is characterized by a large extracellular N-terminal domain of 200 or more amino acids. This domain contains a conserved pair of cysteines, that are likely to be disulfide-linked, in addition to sites for N-linked glycosylation (1). Later in the primary sequence, there are four hydrophobic domains that are predicted to be transmembrane (designated TM1 - TM4), which places the C-terminus also on the extracellular side. Between TM3 and TM4, there is a large, and somewhat variable, intracellular loop which, in some subunits, contains consensus sequences for phosphorylation (21).

So far, thirteen $GABA_A$ receptor subunits have been identified in mammalian brain (reviewed in 6). These have been classified according to sequence similarity into four classes, such that there are six α, three β, three γ subunits and one δ subunit. Members of a single class share approximately 70% amino acid sequence identity, while 30-40% identity occurs between members of different classes. In addition, the mRNA which encodes the $\gamma 2$ subunit undergoes alternate splicing giving rise, in all vertebrate species examined to date, to two forms of the $\gamma 2$ subunit distinguished by the presence or absence of eight amino acids in the large intracellular loop between TM3 and TM4 (22-24). A similar splicing event has been shown to give rise to two forms of the $\beta 2$ subunit in the brains of humans (25) and chickens (26) but not rodents (25-27).

In addition, two subunits, termed rho (ρ) 1 and 2, have been identified. These are clearly related to $GABA_A$ receptors in terms of sequence identity but are primarily found in the retina and appear to have a different pharmacological profile to brain $GABA_A$ receptors (28, 29). Receptors that are expressed from mRNA encoding ρ subunits are insensitive to both bicuculline and benzodiazepines. These pharmacological properties are not altered by co-expression with any of the $GABA_A$ receptor subunits (28, 30). It has therefore been suggested that the ρ subunits form the $GABA_C$ receptor (30-32).

Heterologous expression studies have demonstrated that different subunits can combine to form receptors which display different pharmacological properties. Although homo-oligomeric GABA-gated ion channels can be formed in either the *Xenopus* oocyte or the human embryonic kidney (HEK) 293 cell expression systems, they do not possess the full range of functional characteristics of *in vivo* receptors. In order to produce $GABA_A$ receptors with pharmacological properties that resemble those found in mammalian brain at least an α, a β and a γ subunit are required (33). In particular, the pharmacology of the benzodiazepine site appears to be dependent upon which particular α or γ subunit is incorporated into the receptor (see 6). It should be noted, however, that while there is evidence for the existence of a number of different *in vivo* $GABA_A$ receptors subtypes, not even one native receptor subtype has been unambiguously identified to date. Thus, given the existence of at least 13 subunit genes, there is a possibility of over 500,000 $GABA_A$ receptor subtypes, although current evidence suggests that the real number may be only in the tens rather than the thousands (6, 34). A further complication is our ignorance of the relative importance of these receptor subtypes to particular physiological roles. For example, while the combination of an $\alpha 1$, $\beta 2$ and $\gamma 2$ subunit appears to be the most abundant $GABA_A$ receptor subtype in mammalian brain (see 6), the precise physiological or pathological role of this receptor subtype has yet to be defined.

Of particular relevance to this review is the question of the stoichiometry of the three different subunits (α, β, γ) that are thought to occur in at least some $GABA_A$ receptor subtypes. Based upon homology with the better characterized nAChR and electron microscopic studies (see above) it is now believed that the $GABA_A$ receptor exists as a pentamer (20). There is conflicting evidence from different experimental approaches as to the stoichiometry within the receptor complex (35-37). Further, it is not clear how the subunits are oriented within the pentameric configuration. Clearly, such information is necessary for a full understanding of the nature of agonist and modulator binding sites. While the stoichiometry remains controversial, certain site-directed mutagenesis studies described below have provided some further insight, although they were not specifically designed to address this issue.

In this review, we focus on recent studies directed towards identification of structurally important domains in the $GABA_A$ receptor. We consider primarily the insights that have been provided by using mutagenesis techniques. These results are interpreted in the context of current knowledge of the structure and function of this family of ion channels, which has largely come from detailed multidisciplinary studies of the well characterized nAChR.

As discussed below, molecular biological methods have been widely used in attempts to define functional domains of the $GABA_AR$ and other LGICs. The use of domain swapping techniques and site-directed mutagenesis has provided some limited understanding of structural motifs in the $GABA_AR$ that may be involved in ligand binding, ion channel formation, and post-translational modification. Although these are powerful approaches, their use in the analysis of complex, oligomeric, membrane-bound proteins suffers from a number of potential problems. It is, therefore,

important to first comment on some of the problems that arise in the interpretation of mutagenesis experiments.

3. MUTATIONAL ANALYSIS OF LIGAND-GATED ION CHANNELS: GENERAL CONSIDERATIONS

The common approach in mutagenesis studies is to express wildtype or mutant receptors in suitable cell lines or in *Xenopus* oocytes and to characterize receptor properties by radiolabelled ligand binding or electrophysiological techniques. In our experience, a frequently encountered problem is that the introduction of mutations into one or more subunits of the receptor oligomer leads to dramatic changes in the level of receptor expression. Even very conservative amino acid substitutions may greatly reduce, or even eliminate, the expression of receptor at the cell surface with protein being accumulated instead in the endoplasmic reticulum (e.g. 38). Such a profound effect of what are presumed to be small changes in protein structure is perhaps not surprising given the complexity of folding and assembly of large multisubunit transmembrane proteins. This intrinsic complexity has been illustrated in studies of nAChR expression which have shown that only a small fraction of the total protein that is synthesized is correctly assembled as functional receptors (for review, see 39). Thus, until a complete understanding of the mechanisms which govern expression and assembly of complex proteins is obtained, the approach of mutagenesis remains essentially a "hit or miss" procedure.

A related problem in some published mutational studies is a failure to determine if an absence of detectable current or measurable radioligand binding is due to a lack of receptor expression or to a much reduced affinity for the ligand. To distinguish between these possibilities, it is necessary to determine levels of expression using an appropriate technique such as Western blotting carried out in conjunction with detailed studies of receptor-ligand interactions.

Another difficulty associated with mutational analysis of LGICs is the determination of the precise receptor property that is altered. Although radiolabelled ligand binding and electrophysiological experiments are routinely used to study mutant receptor properties, most mutational studies are restricted to the use of only one or other of theses types of analysis. Radioligand binding has the advantage that no specialized equipment is needed and information can be obtained relatively quickly. Although this technique can reveal changes in the apparent K_d or K_i values for specific ligands resulting from the introduction of mutations, it has the limitation that normally only high affinity sites that are present at equilibrium can be measured. Frequently, receptors are characterized by radiolabelled antagonist binding which provides information only on the inactive, blocked state of the receptor. Furthermore, $GABA_A$ receptors, like other members of this ion channel family, become desensitised upon prolonged exposure to agonists. Thus, equilibrium studies of radiolabelled agonist binding provide information only on the desensitised state of the receptor. Usually, therefore, equilibrium radioligand binding studies alone are unable to reveal the functional impact of a mutation.

In order to investigate the effects of mutation on receptor function, a whole cell patch clamp study is often the method of choice. Normally, this technique is used to determine EC_{50} values for various ligands, with a shift in EC_{50} being interpreted as a change in the properties of the mutant receptor. However, the EC_{50} value is a complex function of several variables, including the rate constants for agonist association and dissociation, and the kinetics of channel opening and closing. Since a change in EC_{50} may result from an alteration in any one of these properties, it is not possible to identify the precise molecular event that has been affected by the mutation. Single channel analysis overcomes some of these limitations by permitting detailed analysis of gating mechanisms. However, this technique also has the shortcoming that the interpretation of the mutation's effect is, to a large extent, model dependent. Thus, the accuracy of the model used to describe the various states of the receptor is critical in obtaining meaningful data.

The points listed above dictate that caution must be exercized when interpreting the effects of mutations on receptor properties. Because electrophysiological and radioligand binding studies generally examine two different states of the receptor (the resting "active" state and the equilibrium ligand-bound state respectively), crucial information might be missed if a mutant is analyzed by only one of the two techniques. To obtain a more complete view of the impact of a mutation, it is obviously preferable to use both approaches. Even then, an important caveat remains with mutational analysis of all such complex membrane-bound proteins. Since none of these receptors has, thus far, proved amenable to crystallization, no high resolution structural information is available. Specific mutations may thus have unpredictable consequences and experimental data are subject to serious misinterpretation. At the present time, we can, however, hope for consistency of results and a merging of information from different approaches. We can then begin to develop models of receptor structure and function which will undoubtedly need refinement as better structural data become available.

4. MUTATIONAL ANALYSIS OF $GABA_A$ RECEPTOR BINDING SITES

4.1. The Nicotinic Acetylcholine Receptor: A Receptor Prototype

The nAChR is the best characterized of the LGICs, due largely to its abundance in the electric organs of *Torpedo* species. Over the last twenty years, a wealth of information about the nature of the ligand binding domains of this receptor

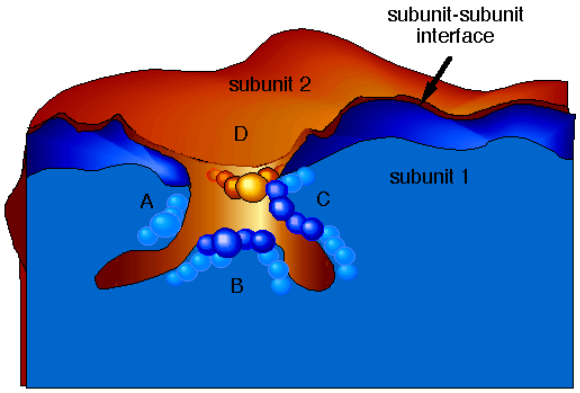

Figure 1 Schematic illustration depicting the hypothetical arrangement of the four loops proposed to form the ligand-binding pocket at subunit-subunit interfaces in members of the LGIC family of receptors. Loops A to C on subunit 1 and loop D on subunit 2 represent the four loops that have been demonstrated by multi-disciplinary approaches to contain residues necessary for ligand-receptor interaction (see Figure 2 and text for details). Subunit 1 in the foreground is shown in cross-section to reveal the contribution made by subunit 2 to the binding pocket at the subunit-subunit interface.

has been accumulating from multidisciplinary studies. Several excellent reviews on this subject have information from the nAChR studies, pertinent results from these earlier experiments will be discussed very briefly here.

One of the more widely discussed models proposed to explain the structure of the ligand binding domains of nAChR is the loop model developed by Changeux and colleagues (see 41). By integrating information from affinity and photoaffinity labelling studies, protein sequencing, receptor expression and mutational analysis, this model proposes that each of the two high affinity ligand binding sites is made up by four distinct, discontinuous protein loops. Three of these loops (termed A, B and C) occur within the large extracellular N-terminal of the α subunit, and the fourth (loop D) is part of the extracellular domain of a neighbouring α or δ subunit. Specific amino acids that have been identified in each of these putative loops are illustrated in Figure 1. The emerging theme is that the high affinity sites for agonists and competitive antagonists lie at the interfaces between the α-γ and α-δ subunits. Since the interfaces are non-identical, this model can explain the non-equivalence that is seen in the binding of some nAChR ligands, such as d-tubocurarine and α-conotoxin (42-46).

Based on much previous work on multisubunit enzymes (47) and the evidence supporting the above model for ligand binding sites in nAChR, it may be predicted that the occurrence of binding sites at subunit-subunit interfaces is a common feature of multimeric proteins. As described below, this model appears to be relevant also to the location of binding sites in the $GABA_AR$ (and also the GlyR). Most of the residues identified to date as important determinants for the recognition of GABA and benzodiazepines lie within one of the four loops predicted by the above nAChR model.

4.2. GABA Recognition Site(s)

4.2.1. Residues implicated in GABA binding

The first hints concerning the location of the GABA binding site on the $GABA_A$ receptor were obtained from biochemical studies. In early photoaffinity labelling experiments, the β subunit was identified as the major site of incorporation of the GABA analogue, [^{3}H]muscimol (12, 13). The β subunit is not, however, the only determinant of agonist binding. In expression studies, it has been shown that individual α, β, γ and δ subunits are capable of forming homo-oligomeric GABA-gated chloride channels when expressed alone *in vitro* (48, 49). This suggests that most, if not all, $GABA_AR$ subunits can form functional GABA binding sites which, when occupied by agonists, mediate channel opening. However, since the properties of the homo-oligomeric channels are not entirely consistent with the native receptor, it is likely that the contributions of residues from other subunits are important for conferring the properties of native receptors.

Mutational analysis of the β subunit has provided evidence for the involvement of tyrosine and threonine residues in GABA recognition. Using rat clones of α1, β2, and γ2 subunits expressed in *Xenopus* oocytes, Amin and Weiss identified two domains in the β subunit which appear to be crucial for the activation of the receptor by GABA (50). Their analysis targeted regions of the β2 subunit which correspond to loops B and C of the nicotinic four loop model (see Figures 1 and 2). The two domains consist of inverted repeats of four amino acids, YGYT and TGSY (Figure 2) at positions 157-160 and 202-205, respectively. The conservative mutations Y157F and Y205F each produced a 50-fold increase in EC_{50}, whereas mutations T160S and T202S produced 20-fold increases. Mutation of other residues in these two domains had little or no effect on the EC_{50} for GABA. Less conservative substitutions of the tyrosine residues produced even more dramatic shifts in the EC_{50}, demonstrating the importance of the aromatic ring structure in these positions. The observed effects were relatively specific for the β subunits since similar changes introduced into homologous residues in the α or γ subunits resulted in rather modest shifts in EC_{50} values. As noted above (Section 3), the interpretation of the consequences of mutations is often complex. However, in this study, the authors provide a convincing argument that the mutations affected agonist binding and not channel gating, since it was always possible to overcome the reduction in ligand recognition and reach the same maximum current seen in wild-type receptors by using an increased concentration of GABA (see 51).

	LOOP A		LOOP B		LOOP C	
rat $GABA_A$ α1	92	KIWTPDTFFHNGKK	138	CPMHLEDFPMDAHACPLKFGSYAYTRA	196	TVDSGIVQ-SSTGEYVVMTTHFHLKR
rat $GABA_A$ α2	117	KIWTPDTFFHNGKK	163	CPMHLEDFPMDVHACPLKFGSYAYTKA	221	VVGTEIIR-SSTGEYVVMTTHFHLKR
rat $GABA_A$ α6	91	KIWTPDTFFRNGKK	137	CPMRLVNFPMDGHACPLKFGSYAYPKS	195	TVSSETIK-SNTGEYVIMTVFHLORK
rat $GABA_A$ β2	90	QLWVPDTYFLNDKK	136	CMMDLRRYPLDEQNCTLEIES**YGYT**TD	192	KLITKKVVF-S**TGSY**PRLSLSFKLKR
rat $GABA_A$ γ2	105	KIWIPDTFFRNSKK	152	CQLQLHNFPMDEHSCPLEFSSYGYPRE	206	RNTTEVVK-TTSGDYVVMSVYFDLSR
hum GABA ρ1	131	KIWVPDMFFV**H**SKR	177	CNMDFSRFPLDTQTCSLEIESYAYTED	233	HTTTKLAFYSSTGWYNRLYINFTLRR
hum GlyR α1	92	SIWKPDLFFANEKG	138	CPMDLKNFPMDVQTCIMQLES**F**G**Y**TMN	193	KDLRYCTKHYNTGKFTCIEARFHLER
hum GlyR α2	99	SIWKPDLFFANEKS	145	CPMDLKNFPMDVQTCTMQLESFEYTMN	200	KELGYCTKHYNTGKFTCIE**V**KFHLER
Tor nAChRα	84	DVWLPDLVLYNNAD	128	CEIIVTHFPFDQQNCTMKLGIWTYDGT	185	KHWVYYTCCPD-TPYLDITYHFIMQR
chick nAChR α7	83	LIWKPDILLYNSAD	127	CYIDVRWFPFDWQKCNLKFGSWTYGGW	181	KRTESFYECCK-EPYPDITFTVTMRR

Figure 2. Sequence alignment of putative loops A, B, and C of various ligand-gated ion channels (see text for details). Double-underlined residues: $GABA_A$ α1, α2 and α6, residues important in benzodiazepine recognition; GlyR α1, α2, residues involved high affinity (site I) binding of agonists; nAChR α and α7, conserved aromatic residues demonstrated to be involved in agonist and antagonist recognition. Bold and underlined residues: $GABA_A$ β2, residues involved in GABA recognition; GABA ρ1, residues involved in GABA recognition; GlyR α2, residues involved in forming the low affinity (site II) agonist site.

It is interesting to note that in each of the above two domains identified by Amin and Weiss (50), several amino acids are conserved in nACh, glycine and $5HT_3$ receptors (see Figure 2). In the first domain, Y157 corresponds to W148 of the nicotinic α7 subunit and to W149 in the equivalent position of the *Torpedo* α subunit. These residues have been implicated in agonist recognition by both DDF photolabelling and by mutagenesis (reviewed in 52). Glycine 158 corresponds to G167 of the GlyR α2 subunit, a residue which has been shown to be critical in forming the glycine recognition site referred to as site I by Schmeiden *et al.* (53). The second tyrosine in this domain, Y160, is homologous to Y151 of the *Torpedo* α1 subunit, a residue which is also photolabelled by DDF (reviewed in 52). A homologous residue is present in all $GABA_AR$ and GlyR subunits and, in the GlyR α1 subunit, this residue has been implicated in the discrimination of ligands at the GlyR (54).

In the second domain identified by Amin and Weiss (50), the tyrosine at position 205 is conserved in several nAChR subunits. Photolabelling and mutagenesis studies indicate that this residue plays an important role in ligand binding and possibly also in the coupling of ligand binding to channel gating in the nAChR (reviewed in 52). The homologous position in the $5HT_3$ and GlyR β receptor subunits is also a tyrosine, while in GlyR α subunits, the residue in this position is a phenylalanine. Mutation of this residue in GlyR α subunit results in changes in agonist recognition (55). Thus, it would appear that these two domains are conserved throughout the family of LGICs as important determinants of ligand/receptor interaction.

The findings discussed above illustrate that discontinuous regions of the β subunit are important for GABA binding. As previously noted, the two domains targeted by Amin and Weiss (50) for mutagenesis are homologous to two of the three loops involved in agonist recognition in the nAChR α subunit. In the nAcChR, specific residues in the γ and δ subunits have also been implicated in forming the fourth loop (loop D; see Figure 1). Photoaffinity labelling and protein sequencing studies of *Torpedo* nAChR have shown that the competitive antagonist, [^{3}H]d-tubocurarine is incorporated not only into the α subunits, but also into W55 of the γ subunit and W57 in the homologous position of the δ subunit (56, 57). In early equilibrium binding studies, the two binding sites for d-tubocurarine in nAChR were shown to be non-equivalent, being characterized by a 200-fold difference in affinities (58). Site-directed mutagenesis of these residues has more recently suggested that γW55 contributes to the high affinity binding site for this ligand, while δW57 is involved in forming its lower affinity site (59).

In support of the notion that agonist binding sites occur at subunit-subunit interfaces, a homologous residue to those labelled by [^{3}H]d-tubocurarine in the nAChRγ and δ subunits has been implicated in the agonist binding site of the $GABA_AR$. As discussed below, this residue is found in the α subunit suggesting that the GABA binding site(s) may be located at the β-α interface of this receptor. Sigel *et al.* (60) identified a leucine at position 64 in the rat α1 subunit whereas, in other published sequences, a phenylalanine had been reported in this position (61, 62). When this unique clone was coexpressed with β2 and γ2 subunits in oocytes, the EC_{50} value for GABA was much greater than that reported by others. In addition, the Hill coefficient was close to 1,

suggesting a loss of cooperativity of channel activation. Further investigation of the properties of this unique subunit revealed that its inclusion in receptor oligomers caused profound changes in the responses to the competitive antagonists, bicuculline methiodide and SR95531. Thus this residue in the α subunit appears to play an important role in the recognition of both agonists and antagonists. The mutation of adjacent residues produced only moderate changes in EC_{50} values for GABA. Homologous residues in the β and γ subunits were mutated to leucine, and this substitution resulted in similar, though smaller, shifts in EC_{50} values to the right. Additional evidence for the involvement of αF64 in agonist binding has since come from photoaffinity labelling studies in which this residue in the rat α1 subunit was identified as a site of labelling by [^{3}H]muscimol (63).

The results of the above studies suggest that, as in the nAChR, the agonist binding site(s) for the $GABA_AR$ occurs at subunit-subunit interfaces, specifically between the β and α subunits. Domains in the β subunit of the $GABA_AR$, which correspond to loops B and C of the nAChR four loop model (see above), appear to play a major role in ligand recognition. In addition, a residue (F64) in the α1 subunit of the $GABA_AR$ corresponds to homologous residues in the γ and δ subunits of *Torpedo* nAChR which appear to participate in agonist and antagonist binding (loop D; see Figure 1). By analogy with the nAChR, it may be predicted that the region of the β subunit which corresponds to the A loop of the nAChR α subunit may also contain important determinants for GABA recognition. As yet, there have been no published studies to support this prediction.

4.2.2 Investigation of a role for a conserved disulfide loop

A common feature of LGICs is a disulfide bridge between cysteine residues in the extracellular N-terminal domain of each subunit (reviewed in 1). These cysteines lie within loop B in the ligand binding model illustrated in Figures 1 and 2. This disulfide loop is thought to stabilize the tertiary structure of the extracellular domain and to be important for receptor assembly and expression. In studies of the *Torpedo* nAChR, for example, Sumikawa and Gehle found that the main effect of eliminating the disulfide loop from either the α or β subunit was retention of the altered subunits in the cytoplasm of the oocytes in which they were expressed (64). However, there was also some indication that the disulfide bridge may have been involved in the formation of the α-bungarotoxin binding site.

Since the disulfide loop is so highly conserved, this feature was modelled by Cockcroft *et al.*(65) as an important determinant in ligand recognition by all members of the receptor family (see Section 7). In testing this model, however, Amin *et al.*, using rat cRNAs expressed in *Xenopus* oocytes, showed that mutations within this loop did not significantly affect ligand interaction with the $GABA_AR$ (66). They specifically targeted the residues in the β subunit that were proposed to form electrostatic interactions with the charged groups present in the GABA molecule, and substituted them with either a neutral residue or a residue of the opposite charge. Using numbering that refers to the position of residues within the cystine loop, they showed that substitution of arginine at position 6 (proposed to interact with the negative site of GABA) caused a moderate (3-fold) shift in EC_{50} values to the right. Since this effect occurred regardless of the charge of the introduced amino acid, this residue is unlikely to be involved in electrostatic interaction with GABA. Substitution of the tyrosine residue at position 8 (proposed to confer selectivity for GABA) with phenylalanine produced a channel that was approximately 3-fold more sensitive to GABA activation, while substitution with a serine resulted in no detectable current, probably due to deficits in receptor expression. Mutation of the residue D11 (proposed to interact with the positive amino group of GABA) produced two results. Substitution with asparagine resulted in no current, while substitution with glutamate did not affect the EC_{50}, but did decrease the E_{max} and Hill coefficient. The authors suggest that these effects are a result of general disruption of receptor assembly and/or gating. The most dramatic change was seen when L5 was substituted with glutamine. This mutation resulted in a twelve-fold increase in the EC_{50} value for GABA, and a decrease in maximum current.

Removal of the cysteine residues which form the disulfide bridge invariably led to a lack of GABA-gated currents, presumably due to much reduced receptor expression (66). In this study, the authors were able to discriminate between loss of expression and lack of agonist recognition by introducing the same mutations into the γ subunit. Coexpression of the mutated γ subunit with and β produced GABA-gated channels which had an EC_{50} closely matching that obtained when α and β subunits alone were expressed. Further evidence that the mutated γ subunit was poorly expressed, if at all, was that these receptors lacked characteristics normally attributed to the γ subunit *i.e.* sensitivity to zinc and to the benzodiazepine agonist, diazepam. Thus mutational analysis of the conserved disulfide loop suggests that the loop is more important in the expression and assembly of the receptor than in agonist recognition.

4.2.3 A role for aromatic and hydroxylated amino acids in GABA recognition

The above studies show that, to date, few residues have been identified as being involved in GABA recognition. Those that have been implicated in binding tend to be either aromatic or polar in nature. One feature that is shared by the endogenous ligands of all members of the LGIC family (nAChR, $GABA_AR$, GlyR, $5HT_3R$) is a positively charged nitrogen at physiological pH. In early studies, it was reasonably predicted that this positive charge would be stabilized by an acidic amino acid residue (aspartate or glutamate) within an anionic subsite of the receptor. However, this idea was challenged when the three-dimensional structure of acetylcholinesterase, at 2.8 Å resolution, showed that the quaternary ammonium moiety of ACh was bound, not to a negatively charged site, but rather to a preponderance of aromatic residues lining a deep gorge forming the binding site (67). Since then, evidence has been accumulating to suggest that aromatic residues are involved in the agonist binding domains of the nAChR, GlyR and $GABA_AR$.

In the case of the nAChR, aromatic residues have been suggested either to form a negative subsite in the binding pocket which interacts with the quaternary ammonium group of ACh or to be involved in hydrophobic interactions with this portion of the ligand (reviewed in 40, 68). It has also been suggested that the most important factors for ACh recognition are the hydroxyl groups of the highly conserved tyrosines in the nAChR binding site (69). In the case of the $GABA_AR$, the hydroxyl groups of residues Y157 and Y205 appear to be important in ligand recognition, but perhaps not as important as the aromatic side chain itself (50). The size of the residue and the presence of a hydroxyl group at position 202 of the β-subunit appears to be essential in mediating GABA recognition. Substitution of this residue with serine shifted the dose response curve significantly, while substitution with alanine produced receptors that, although they did not respond to GABA, could be directly activated by pentobarbital, indicating that these mutations did not compromise receptor assembly (50). The phenylalanine (F64) in the α1 subunit that has been suggested to be important in agonist recognition (see above) has, thus far, been substituted only by a leucine in a natural mutation (60). Thus, the chemical specificity for ligand recognition in this position is unknown.

As yet, although potentially important amino acid residues within the receptor have been identified, there is no good experimental evidence to indicate which parts of the GABA molecule are recognized by these residues. However, it is likely that, by analogy to the nAChR, the hydroxyl and aromatic groups of the residues identified by Amin and Weiss (50) and Sigel *et al.* (60) may interact with the positively charged portion of the GABA molecule.

4.2.4 Other GABA binding proteins

Analysis of the GABA recognition sites in other GABA-binding proteins may provide further information on requirements for the recognition of this molecule. The GABA binding site of the GABA receptor formed by the ρ1 subunit has been examined using site directed mutagenesis. As noted in section 2, receptors formed by ρ subunits have properties that distinguish them from the $GABA_AR$ subunits. Of seventeen mutations made within the proposed extracellular N-terminal domain of the ρ1 subunit, only three produced significant changes in the response of the receptor to GABA as measured by electrophysiology (70). The largest change in the response to GABA came as a result of the mutation Q189H, which produced an 11-fold decrease in GABA potency, and a reduction in the Hill coefficient. This residue lies within the di-cysteine loop which is common to all LGICs (see Section 4.2.2). Further analysis prompted the authors to suggest that the effects of this mutation could be due to a change in the allosteric coupling between binding sites, and not necessarily to a change in agonist recognition. Substitution of a histidine at position 141 with an alanine also reduced the response of the receptor to GABA by three-fold and decreased the maximum current, but left the Hill coefficient unaltered. The authors conclude that the histidine residue is either involved directly in agonist binding or in stabilizing the conformation of the binding site. However, since the relationship between agonist binding and channel activation is unknown, this conclusion may be premature.

Tryptophan residues appear to play a crucial role in the agonist recognition site of the GABA transporter, GAT-1. One of these tryptophan residues is conserved throughout all identified amino acid transporters, and, as with the LGICs, has been proposed to interact with the amino group of their amino acid substrates (71). A model of the active site of GABA aminotransferase, the enzyme responsible for GABA degradation in the CNS, has been proposed (72) in which the carboxylate group of GABA interacts with an arginine and a lysine residue. It is possible that similar, as yet unidentified, residues may exist in the GABA binding pocket of the $GABA_AR$ that interact with this portion of the agonist.

4.3. Benzodiazepine Recognition Site(s)

4.3.1. Importance of the alpha subunit(s)

An understanding of the manner in which the benzodiazepines interact with the $GABA_AR$ has been of great interest because of the clinical importance of these compounds. Initial studies of native receptors (see above) revealed a heterogeneity in benzodiazepine binding that was thought to result from the presence of more than one type of site for these drugs in the mammalian brain. Based on differential affinity for the specific ligand, CL218-872, two pharmacologically distinct receptor subtypes, BZI and BZII, were postulated to exist (for review, see 8). More recently, cloning of multiple $GABA_AR$ isoforms of several different subunit classes, and the subsequent expression of different combinations of subunits in heterologous expression systems has confirmed that the differences in native receptor pharmacology can be attributed to the presence of multiple receptor subtypes (for review, see 6).

Early photoaffinity labelling studies suggested that the major site of incorporation of [^{3}H]flunitrazepam in bovine brain was the α subunit(s) of the $GABA_AR$ (11, 73). This has since been confirmed using subunit-specific antibodies (74, 75). By peptide sequencing of a fragment of photolabelled receptor, the specific site of [^{3}H]flunitrazepam incorporation within the bovine $GABA_AR$ α1 subunit has recently been demonstrated to be a histidine residue in position 102 (76). As will be discussed below, mutational analysis of the $GABA_AR$ had previously suggested the importance of this and other residues in forming the benzodiazepine binding domain.

One of the first studies to identify specific amino acid residues within the α subunit that are involved in benzodiazepine binding was that of Pritchett and Seeburg (77), who investigated the specific determinants of type I and type II benzodiazepine pharmacology. Heterologous expression studies had previously shown that $GABA_ARs$ containing an α1 subunit display a type I BZ-binding profile, while those containing either α2 or α3 subunits display BZII-type pharmacology. Pritchett and Seeburg (77) constructed a series of α-subunit chimearas consisting of sections of the α1 and α3 subunits and, after coexpression with β2 and γ2 subunits in HEK293 cells, assessed their benzodiazepine binding profiles. This approach, coupled with site-directed mutagenesis, led to the identification of a single glycine residue at position 225 of the α1 subunit which, if mutated to a glutamate (the residue

which occupies the homologous position in the α3 subunit), produced a receptor that displayed a BZII-type pharmacology. Unfortunately, no functional assessment of these mutations has been carried out.

Although benzodiazepines are not receptor agonists but rather are modulators of GABA-gated currents, there is evidence to suggest that the benzodiazepine site shares homology with the agonist site, but lies at a different subunit-subunit interface (see 78). In addition to further information cited below, the glycine residue (G225) identified by Pritchett and Seeburg (77) is homologous to Y190 of the *Torpedo* nAchR α subunit. A number of mutational analysis studies have suggested that this residue is involved in forming the agonist recognition site (79, 69) and may be involved in both binding and gating (80). Others have instead suggested that this residue may be involved in the coupling of ligand binding to channel gating and is not directly involved in the initial binding step (81).

As noted above His-102 of the bovine $GABA_AR$ α1 subunit has been identified as a site of photolabelling by [^{3}H]flunitrazepam (76). The homologous residues in rat α subunits appears to be critical in discriminating between the benzodiazepine agonist, diazepam, and the partial inverse agonist, Ro15-4513. Heterologous expression studies have shown that α6 subunit-containing receptors do not recognize the classical benzodiazepine agonist, diazepam, whereas all α subunits (α1- α6) recognize the partial inverse agonist Ro15-4513. To investigate the molecular basis for this specificity, a series of chimaeras between the α1 and α6 subunits was made (82). The chimaeric α subunits were coexpressed in HEK293 cells with β2 and γ2 subunits and their benzodiazepine binding profiles were determined. The specificity of agonist recognition was narrowed down to a single amino acid residue at position 101 *i.e.* the same residue to that which was identified in direct photolabelling studies (see above). The residue in this position is a histidine in the α1 subunit and an arginine in the α6 subunit. Exchange of the α1 subunit histidine for arginine produced a receptor that displayed high affinity [^{3}H]Ro15-4513 binding which could not be displaced by diazepam, CL218-872 or zolpidem. There was also a 200-fold decrease in the affinity of the receptor for the benzodiazepine antagonist, Ro15-1788. The converse occurred in receptors containing α6 subunits in which the arginine had been replaced by histidine. These receptors gained sensitivity to diazepam, Cl218-872, and zolpidem, although the ability of these ligands to displace [^{3}H]Ro15-4513 was reduced relative to wild type α1 subunit-containing receptors. This indicates that, although the residue in the 101 position is a major determinant of benzodiazepine binding, other residues must be involved in forming the agonist binding pocket. Again, no functional studies were performed with the mutated receptors.

Further confirmation of the importance of this α subunit residue in benzodiazepine recognition was obtained when a natural mutation at position 101 of the $GABA_AR$ α6 subunit was found in alcohol non-tolerant rats (83). A glutamine substitution at this position occurred in the α6 subunit of these rats which, when coexpressed with β2 and γ2 subunits in HEK293 cells, produced a diazepam-sensitive receptor. It was further demonstrated that the allosteric effects of GABA at the receptor (inhibition of both [^{3}H]Ro15-4513 and [^{35}S]TBPS binding) were not affected by this substitution. This suggests that the structural requirements for allosteric modulation of the receptor by GABA are not identical to those that mediate benzodiazepine agonist binding.

It is interesting to note that the histidine residue identified in the above studies (H101) resides in a position that is homologous to Y93 of the *Torpedo* nAChR α subunit. This residue is labelled by acetylcholine mustard and DDF, and mutations introduced at this point have shown it to be involved in the binding of agonists and competitive antagonists (for review, see 41).

A further investigation into the differences between the primary structures of the $GABA_AR$ α1 and α6 subunits and the resulting differences in benzodiazepine pharmacology has identified additional residues which, together with H101 and G225, appear to play roles in forming the benzodiazepine recognition site (84). Four residues in the rat α6 subunit (R100, P161, E199, and I211) were substituted in a stepwise fashion with the corresponding residues present in the α1 subunit (H101, T162, G200, and V212). As the α6 subunit residues were replaced by those of α1, there was a corresponding increase in the ability of diazepam to displace [^{3}H]flunitrazepam from the receptors such that when all four substitutions were in place, the receptors were more sensitive to diazepam than were wild type α1 subunit-containing receptors. The first three substitutions had little effect on the K_i values for Ro15-4513 and Ro15-1788, but the combination of all four produced a 20-fold increase in the K_i for Ro15-4513 while producing only a minor increase (2-fold) for the antagonist.

4.3.2 The importance of the gamma subunit(s)

As discussed above, the $GABA_AR$ α subunit is a major determinant of benzodiazepine binding and specificity. However, the characteristic ability of these ligands to modulate GABA-gated chloride conductances in native receptors is dependent on the presence of a γ subunit (33). Furthermore, pharmacological efficacy is determined by the type of γ subunit that is present (for review, see 85). As yet, only one residue within the γ2 subunit has been identified as being important in benzodiazepine recognition and efficacy (86). This residue was revealed by comparison of discrepancies in two reported sequences of the human γ2 subunit. At position 142, a serine was present in the sequence of Pritchett et al (33), whereas a threonine occurred in the sequence determined by Whiting's group (87). When the threonine was changed to a serine and coexpressed with α1 and β1 subunits, the benzodiazepine pharmacology of the receptor was significantly altered whereas there were no measured changes in responses to GABA or to the other receptor modulators, pentobarbital and alphaxalone. In the mutated receptor, Ro15-1788 and Ro15-4513 (normally an antagonist and partial inverse agonist, respectively) became potent partial agonists. In addition, type-I selective agonists became inverse agonists and there was an increased potentiation by non-selective agonists. The authors suggest

that the effects of the mutation are due to differences in the ligand-induced conformational changes in the receptor rather than to differences in the binding site *per se*. In agreement with this possibility, unlike all other residues so far implicated in GABA and benzodiazepine interactions, threonine 142 does not lie within any of the regions corresponding to the four-loop model of the nAChR, and as yet does not have a functional homologue in any other LGIC.

Unlike the residues that have been implicated in forming the domains of the receptor that interact with GABA, no aromatic residues have yet been identified as being important determinants in benzodiazepine recognition. It is likely that some determinants remain to be revealed. In addition, as in all such studies, residues that have been implicated in binding by mutational analysis do not necessarily interact directly with the ligand but rather may stabilize a particular receptor conformation for which some ligands have greater affinity than others.

4.3.3 Other benzodiazepine binding proteins

Mutational analysis of the peripheral benzodiazepine receptor, a protein which bears no significant homology to the $GABA_AR$ but which does recognize classical benzodiazepine agonists such as flunitrazepam and diazepam, has identified residues which confer high affinity binding for the benzodiazepine Ro5-4864. In this protein, it appears that five amino acids (two threonines, a cysteine, a valine, and an arginine) play an integral role in the recognition of Ro5-4864 (88). It may be hoped that further examination of the recognition sites of other benzodiazepine-binding proteins will lead to a better understanding of the structural requirements for binding of these ligands.

4.4 Identification of a Single Residue Conferring High Affinity Loreclezole Binding

Loreclezole is a broad spectrum anticonvulsant agent that acts at the $GABA_AR$. This compound is the only known β subunit-selective agent, with a more than 300-fold greater affinity for β2/β3- than for β1 subunit-containing receptors, a selectivity that occurs independently of the presence of specific α and/or γ subunits (89). To identify the region of the β subunits responsible for loreclezole sensitivity, chimaeras of β1 and β2 subunits were constructed, and it was found that the region between K237 and G334 in the β2 subunit was necessary for loreclezole potentiation of the GABA response in oocytes (90). Mutational analysis of this region identified a single residue, N289, as being responsible for loreclezole sensitivity. The location of this residue is unusual in that it occurs in the second putative transmembrane domain, TM2 (see Section 5), a region of the receptor thought to form the lining of the ion channel. However, the authors point out that the mutated residue, rather than being part of a binding site, may affect the manner in which the binding of loreclezole modulates the gating of the channel. One might suspect the latter to be the case, since, unlike compounds which are known to directly interact with this region of LGICs, loreclezole potentiates the GABA response rather than blocks it.

5. MUTATIONAL ANALYSIS OF THE CHANNEL FORMING REGION

Hydropathy plots of each LGIC subunit reveal four conserved hydrophobic domains that are predicted to be transmembrane (14-17). Of these four regions, the second transmembrane domain, referred to as TM2, appears to form the lining of the ion channel. Extensive photolabelling studies and mutational analysis of the nAChR have identified this region as being involved in ion channel formation (for comprehensive reviews see 91, 92). Furthermore, synthetic peptides corresponding to the TM2 domain of the nAChR (93), and more recently of the GlyR α subunit (94), have been shown to form ion channels in lipid bilayers.

A recently developed technique, the substituted cysteine accessibility method (SCAM), has been used to identify amino acids that are exposed to the hydrophilic lumen of ion channels (95). This technique is based on replacing amino acids in the putative channel forming region with cysteines and then looking at the accessibility of the residues to modification by sulfhydryl-reactive agents. It is assumed that any residue that is exposed to the channel lumen will be accessible to the modifying agents, and modification will be detected as a change in the channel properties of the receptor. Furthermore, by examining the interval of the residues that are exposed to the modification agents, the secondary structure of the region may be predicted

Application of SCAM to the $GABA_AR$ has identified likely channel-lining residues in the rat α1 subunit (96, 97, 98). Residues found to be accessible to cysteine-modifying agents correspond to some of those similarly identified by this technique in the nAChR α subunit (95, 99; see below and Figure 3). Residues V257, T261, T268 and I271, which lie towards the extracellular end of the TM2 domain were shown to be accessible to modifying agents in both the presence and absence of agonist, conditions which are presumed to reflect the closed and open state of the channel respectively. This observation suggests that the channel "gate" must lie deeper in the lumen than residue 257, which is deeper into the channel than had previously been suggested from the results of mutational studies of the nAChR. Coapplication of picrotoxin and GABA protected the mutated V257 from modification. The authors suggest that this may be a result of steric occlusion of the channel by picrotoxin, or less likely, from picrotoxin exerting an allosteric effect on the channel from a distant site.

The accessibility of modified residues to the cationic sulfhydryl reactive agent methanethiosulfonate ethyl-ammonium (MTSEA+) has suggested that the ion selectivity filter must lie deeper into the channel than residue 261. This contradicts earlier models in which the filter had been proposed to be formed by rings of negatively charged residues predicted to be present at the mouth of the channel (reviewed in 91; see Section 7). However, the SCAM data are consistent with the results of Galzi *et al.* (100) who found that substitution of the negatively charged residues in the putative intermediate and outer rings of the nAchR with neutral amino acids did not change the ion selectivity of the receptor. It is

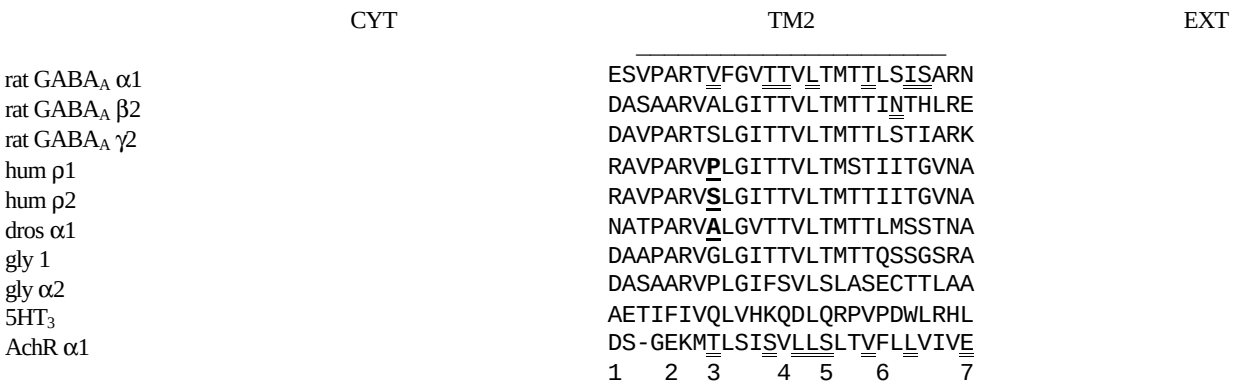

Figure 3. The TM2 region of several ligand gated ion channels. CYT, cytoplasmic face; EXT, extracellular face. Double-underlined residues: 1, identified by SCAM as present in the channel lumen; 2, residue conferring high affinity loreclezole binding. Bold and underlined residues: 1 and 2, residues conferring Picrotoxin sensitivity to rho receptors; drosophila 1, residue changed to a serine in insecticide-resistant strains.
Numerals correspond to the following : 1. Inner ring, 2. Intermediate ring, 3. Threonine ring, 4. Serine ring, 5. Leucine ring, 6. Valine ring, 7. Outer ring

interesting that, in the latter study, one of the most important determinants of charge selectivity was shown to be the length of the linker between TMI and TM2, a stretch of amino acids that is predicted to be intracellular

.

Investigation of the nAChR ion channel by SCAM has identified several residues in the TM2 region of the α subunit that are likely to be involved in its formation (see Figure 3). Initially, this method produced a pattern of cysteine modification in the nAChR subunit that was believed to be consistent with a β-sheet structure for TM2 (95). However, further analysis suggested that this region exists as an α-helix interrupted by three amino acids in an extended β-strand structure (99). A similar structure has now been proposed for the TM2 region of the $GABA_AR$ α1 subunit, with the interruption of the α-helix occurring in the region of T262 (98).

Examination of GABA receptors formed from ρ1 and ρ2 subunits points to one particular amino acid in the TM2 region of these subunits playing an important role in determining sensitivity to picrotoxin (101). When expressed in *Xenopus* oocytes, homomeric receptors formed by ρ1 subunits are approximately ten-fold more sensitive to picrotoxin than their ρ2 counterparts. The Hill coefficients of 1 for the ρ1 subunit homomeric receptor and 2 for the ρ2 subunit receptor, suggested that picrotoxin binding is non-cooperative in the former case and cooperative in the latter. Construction of chimaeras between these two subunits showed that the structural element conferring both the increased sensitivity of the ρ2 subunit to picrotoxin and the change in Hill coefficient occurs at position 309 in the TM2 domain. At this position there is a proline in the ρ1 subunit and a serine in the ρ2 subunit. This residue occurs in the homologous position to V257 of the rat $GABA_AR$ α1 subunit identified using SCAM, and an alanine in the Drosophila α subunit which, when replaced by a serine in insects, confers resistance to picrotoxin and cyclodiene insecticides (102).

As for nAChR and other LGICs, mutational analysis of the TM2 region of the $GABA_AR$ provides strong evidence for this domain being part of the ion channel. Comparison of the primary sequences of various LGICs reveals conservation of residues both in TM2 and in the regions flanking this domain. Thus, the channel structure itself seems to be highly conserved even though some channels conduct cations, while others are anion selective. While a great deal of evidence points to the importance of the TM2 domain in channel formation, there is also evidence that regions other than TM2 are involved. Substitution of cysteine residues in the TM4 region of the nAChR, for example, can result in dramatic changes in the properties of ion conductance (103-105), suggesting that the overall conformation of the protein and its interaction with the lipid environment may also influence the properties of the ion channel.

6. MUTATIONAL ANALYSIS OF POST-TRANSLATIONAL MODIFICATIONS

6.1 Phosphorylation sites

The $GABA_AR$, like several other LGICS, can be phosphorylated by various protein kinases (for reviews see 21, 106). Analysis of the primary sequences of the $GABA_AR$ β and γ subunits reveals that, within the presumed intracellular loop between TM3 and TM4, there are consensus sequences for phophorylation by cyclic AMP-dependent kinase (PKA), calcium-calmodulin-dependent kinase (PKC), and tyrosine-protein kinase (PTK). Biochemical studies have revealed that both the β and γ subunits can be phosphorylated by PKA *in vitro* (107, 108) and that the γ subunit can also be phosphorylated by PKC (109). The functional consequences of phophorylation appear to vary, however, in a tissue-dependent

manner. In general, phosphorylation leads to changes in the amplitude, frequency, and desensitization rates of GABA-gated currents.

By using a combination of phosphopeptide analysis and site directed mutagenesis, the phophorylation sites of the β and γ subunits have been definitively identified. Phosphopeptide analysis of the α1β1γ2 receptor subtype expressed in HEK293 cells demonstrated that phophorylation by PKA occurred exclusively on serine residues of the β1 subunit (110-112). Mutational analysis of these residues in the intracellular loop of the β1 subunit identified a serine at position 409 as being the substrate for PKA. Replacement of serine with an alanine resulted in a complete loss of phosphorylation, and prevented modulation of GABA-gated currents by PKA.

A similar approach was taken to identify regions of the β and γ subunit that are phosphorylated by PKC (109-112). Mutational analysis showed that PKC phophorylated the same serine in the β1 subunit intracellular loop as PKA and, as might be expected, produced the same functional consequences. Both γ2S and γ2L subunits are phosphorylated by PKC at position 307 in the large intracellular loop. The long and short forms of the γ2 subunit differ by the absence or presence of a sequence of 8 amino acids within the intracellular loop. Within this sequence in the γ2L subunit, there is an extra PKC phosphorylation site at position 343. The sensitivity of the $GABA_AR$ to ethanol has been shown to be dependent on phophorylation of this residue (113, 114). Phosphorylation of either of the two PKC sites in the γ2L subunit produced slightly different regulation of GABA-gated current than was seen when only the β subunit was phosphorylated.

6.2 Glycosylation sites

Glycosylation appears to be an important facet of the expression and assembly of LGICs, although, at least for the nAChR, it may not be an absolute requirement for cell surface expression (115, 116). The only mutational analysis of this post-translational modification in the $GABA_AR$ introduced glutamine residues in the place of the asparagine residues proposed to form N-linked glycosylation sites in the hydrophilic N-terminal domain of the rat α1 subunit (117). The substitution of either or both arginines at position 10 and 110 formed a $GABA_A$ receptor with normal pharmacology when coexpressed in *Xenopus* oocytes with β1 and γ2 subunits. The whole cell current produced by these mutant receptors was not significantly different than that seen with wild-type receptors, indicating not only that glycosylation was not important for function, but that lack of glycosylation probably did not impair receptor expression. A different picture emerged when the mutated subunit was expressed with β1 and γ2 subunits in HEK293 cells. In this system, expression of either the N10Q or N110Q mutations at 37°C resulted in significantly reduced expression levels as measured by [^{3}H]Ro15-1788 binding. Furthermore, the expression level of the double mutant was such that no measurable binding of [^{3}H]Ro15-1788 was detected. Since the K_d values for these mutants were similar, the reduced level of radioligand binding was due to differences in expression levels. Thus, in the HEK293 expression system, appropriate glycosylation is a major component in subunit assembly and expression. Interestingly, the processing of non-glycosylated subunits appears to be temperature dependent. When mutant receptors were expressed in HEK293 cells at 30°C, some of the expression returned to wild type levels.

7. RECENT MODELS OF $GABA_A$ RECEPTOR STRUCTURE AND FUNCTION

This section will briefly overview some of the models that have been proposed to explain the ligand binding and channel opening properties of the $GABA_AR$ and other LGICs. As described above, a combination of photoaffinity labelling studies and mutational analysis has led to the assignment of TM2 as a channel lining structure. Based on the results of nAChR studies, a theoretical model of the ion channel was proposed (118). This model postulated that the channel is made up by a series of amino acid rings, each composed of specific types of residues projecting from the α-helical TM2 region of each subunit into a central lumen. It was further suggested that specific rings of amino acids underlie the ion and charge selectivity of the channel (119). However, further mutational analysis of the TM2 regions of LGICs has provided contradictory data, particularly with respect to the location of the charge selectivity filter and gate (see Section 5). Furthermore, there is also evidence that the TM2 region may not be completely α-helical in nature, but may be made up of both α-helical and β-sheet structures (120).

An early model of neurotransmitter binding sites by Cockroft *et al.* (65) was based on the molecular modelling of the conserved cystine loop in the extracellular N-terminal domain. This region was examined because the loop structure is conserved throughout the known LGIC family and because several residues within this loop are either invariant or homologous. Thus, it was reasoned that the loop functions as a prototypical ligand binding domain for this superfamily of receptors, with ligand specificity arising from the presence of unique residues. The loop was modelled as a rigid β-hairpin. Residues that were proposed to interact with GABA were a conserved aspartate residue (D11) proposed to interact with the positively charged amino group and a conserved aromatic group at position 8 (phenylalanine in α1 and γ2, tyrosine in β2) proposed to interact with the negatively charged carboxyl

moiety. The residue at position 6 of the loop was proposed to be the residue conferring the specificity of the ligand interaction. However, as described above (Section 4.2.2), mutational analysis of this region in both the $GABA_AR$ and nAChR showed that the presence of this loop is more important for the correct assembly of the receptor in the cell membrane than for ligand recognition. It is, therefore, unlikely that the residues proposed in the molecular modelling study actually interact with the charged groups of the GABA molecule.

A model of the ligand binding domains of GABA and benzodiazepines has been proposed by Smith and Olsen (78) based on both biochemical data and mutational analysis. They propose the existence of two homologous binding domains for GABA and benzodiazepines within a single α subunit. Each structural domain is formed by two β-sheets separated by an α-helical region. Each β-sheet region contains a TXDXFF domain, and includes residues that have been photoaffinity labelled by either [^{3}H]muscimol or [^{3}H]flunitrazepam, or have been implicated in ligand binding by mutational analysis. This model suggests that the binding of ligand induces conformational twists in the α-helical segment which are then transduced to the ion channel. The binding of benzodiazepines to the second site was suggested to modulate the degree of twisting induced by the binding of ligand at the GABA site, thus allosterically modulating GABA-induced chloride flux. This model takes into account the probable involvement of residues of adjoining subunits in forming the binding site and proposes that, as has been suggested for the nAChR, ligands bind at subunit-subunit interfaces. The residues proposed by Smith and Olsen (78) to form the agonist site include loop A and residues N-terminal to this domain, in addition to putative loop D of the nAChR model. The β-α-β model does not, therefore, include the residues in loops B and C that Amin and Weiss (50) identified as being important for GABA recognition. In addition, there is evidence to suggest that several determinants for benzodiazepine efficacy lie further C-terminal to the binding domains proposed in the model (see Section 4.3).

Aprison and colleagues have also recently described models of agonist binding sites (121, 122). Their computer modelling approach used information derived from sequence homologies and mutational analysis. In addition to suggesting which residues may be involved in agonist binding, the most recent model (122) proposes a mechanism by which agonist binding induces chloride ion flux. Residues predicted to form the agonist binding site were D146, E155, T202, Y205, R216 and R269. Residues D146, Y205, and T202 are proposed to form electrostatic interactions with the positively charged amine of GABA, while the two arginines form hydrogen bonds with the carboxyl group. E155 is proposed to form a charge-transfer complex with R216, resulting in stronger bonding with the carboxyl group. These interactions then result in channel opening, during which the guanidinium group of R269, proposed to attract Cl^- ions, is pulled to the mouth of the channel. In the same study, a nearly identical model based on homologous residues is proposed for the glycine receptor. Although not explicitly stated within the text, the model presented by Aprison *et al.* (121) deals only with the β-subunit. The residues identified as forming the GABA binding site reside in loops B and C, which is consistent with Amin and Weiss' mutational analysis of the GABA binding site (50). However, R269 which is predicted to line the mouth of the channel, has not previously been identified as an important determinant of GABA binding. This residue has a homologue in the GlyR α subunit at position 219, which mutational analysis has implicated in subunit processing rather than ligand binding (38). Furthermore, D146 that is proposed to interact with the positively charged amino group of GABA has already been shown to have little effect on GABA recognition (66). Only two of the residues (T202 and Y205) have been shown to be important in agonist interaction by mutational analysis (50).

An alternative approach to define the basis for ligand-induced ion channel gating, is to determine the three-dimensional structure of the proteins. So far only very low resolution structural information is available for the $GABA_A$ receptor. However, electron microscopy has shown that, like the nAChR and $5HT_3$ receptor, the $GABA_AR$ exists as a pentamer, with the subunits arranged to form a central ion channel (20). Using cryoelectron microscopy of crystallised *Torpedo* membranes, the structure of the nAChR has been resolved to a resolution of 9Å (19). Analysis of computer enhanced images, suggests that each subunit contains three α-helical rods running perpendicular to the membrane. In two subunits (presumed, though not proved, to be the α subunits), the rods contain cavities which have been suggested to be the ACh binding sites. In an attempt to identify structural differences between the closed and open states, the position of specific regions of the receptor in the presence and absence of agonist has been investigated (123). Unfortunately, in this study, there is no evidence to suggest that the channel had actually opened in the presence of agonist. However, the results have led to the proposal of a model by which agonist binding induces channel opening. Acetylcholine is proposed to bind to the two α subunits, one of which is adjacent to the γ-subunit (designated αγ) and the other borders the δ subunit (designated αδ). In this model, the α subunits are separated by the β subunit, although most current models favour the γ subunit in this position (40). The binding of agonist is considered to induce a twist of the three helical rods in the αδ subunit and, to a lesser extent, in the αγ subunit. The small conformational change in the αγ subunit pulls the relatively rigid β subunit away from the αδ subunit, allowing it to twist

more freely. This is suggested to be the basis for the cooperativity in binding that is seen in channel gating. The region identified as TM2 in each subunit is proposed to line the channel and is α-helical except for a kink about halfway down the lumen. This kink, which may correspond to the non-helical portion of the TM2 domain identified by Akabas *et al.* (99), has been suggested to form the gate in the closed conformation of the receptor. When agonist binds, the resulting conformational change is translated through to the membrane-spanning region causing the helices to rotate. As a result, the kink moves away from facing the center of the lumen, and a region of constriction is formed at the cytoplasmic end of the channel. Unwin suggests that the kink is formed by the bulky side chain of L251 which projects into the channel in the closed state (123). However, this is contradicted by SCAM studies of the nAChR in which the gate is proposed to be at least as cytoplasmic as E241 (99; see Section 5). SCAM also suggests that L251 faces a hydrophilic environment only when in the open state (95), which is again inconsistent with the above proposal.

8. CONCLUSIONS

In recent years, the complexity of the $GABA_A$ receptor, both with respect to subunit heterogeneity and its multiple interacting binding sites, has become apparent. Mutagenesis techniques, combined with biochemical and electrophysiological analysis, are beginning to provide information on structure-function relationships of this important neurotransmitter receptor. By integrating information from studies of the $GABA_AR$ and other members of the LGIC superfamily, initial attempts have been made to model receptor-ligand interactions and their consequences on channel activity (see Section 7). While these models provide a useful framework in which to envision receptor structure and function, none are able to reconcile all of the experimental data on the binding and gating of the $GABA_AR$. At this point, it is impossible to develop a model to explain all of the complex allosteric interactions that occur at this receptor, particularly in view of the sometimes subtle differences in pharmacological properties that are displayed by different $GABA_A$ receptor subtypes. This will ultimately require the elucidation of the complete three dimensional structure of individual receptor subtypes at the atomic level and more extensive characterization of their various ligand binding sites.

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