Molecular Biology of Inhibitory Amino Acid Receptors

*Ronald S. Duman, Paul M. Sweetnam, Peter A. Gallombardo, and John F. Tallman**

Ribicoff Research Facilities, CMHC
Yale University School of Medicine
34 Park Street, New Haven, CT 06508

Contents

Introduction

Biochemical approaches to neurotransmitter receptors have been extensively exploited in recent years. With the introduction of radiolabeled analogs of transmitters or drugs thought to act at these transmitter receptors, it has become possible to directly study the binding sites of neurotransmitter receptors and draw conclusions as to the relative order of potency of drugs at these sites and their putative pharmacological properties. In addition, many conclusions about the properties of the neurotransmitter receptors have been reached by comparing the equilibrium binding constants and apparent numbers of receptors following various pharmacological and physiological manipulations. Although some of these conclusions may be justified, many investigators do not possess the expertise to study the more sophisticated aspects of protein structure and molecular dynamics. One unfortunate result of this is that each time an unexplainable break in a binding or displacement curve occurs, a new receptor is "discovered," leading to a generalized confusion in some areas of neurotransmitter research. One has an overall sense that these areas will continue to circle around the core issues until a more sophisticated generation of researchers has broken with the past.

In contrast, in the last 10 yr, research into the mode of action of the minor tranquilizers and related drugs has solidified behind a number of unifying hypotheses that possess value for the development of new compounds with predictable pharmacological properties. In addition, this research has led to a rather sophisticated view of the actions of these compounds and to identification of their sites of action at a receptor site for γ–aminobutyric acid (GABA), one of the brain's major inhibitory neurotransmitters. In spite of very limited amounts of receptor molecules available, advances in molecular approaches to their study have led to the isolation of this receptor complex, preparation of monoclonal antibodies against it, and cloning of genes coding for two of the major subunits of the complex. In this review we will discuss two major areas: first, protein structure as it relates to the function of membrane ionic channels and the kinetic and equilibrium binding constants of transmitter and drug-binding sites; second, how these general principles apply to the functional properties of transmitter-controlled anion channels and related proteins.

Index Entries: Inhibitory amino acid receptors; amino acid receptors; receptors, of inhibitory amino acids; GABA/benzodiazepine/chloride channel; molecular topology, of inhibitory amino acid receptors; patch clamp studies, of inhibitory amino acid receptors; affinity studies, of inhibitory amino acid receptors; cloning, of inhibitory amino acid receptors; glycine/strychnine/chloride channel.

Aspects of Protein Structure Important for Function in the Membrane

Structural levels of organization have been understood for soluble proteins for many years, and recent investigations of membrane proteins have relied on these concepts. Studies of model membrane proteins, such as bacteriorhodopsin and (more recently) voltage-dependent and ligand-gated Na^+ channels, have further indicated the generality of these principle.

Three-dimensional studies have demonstrated the uniqueness of a general folding pattern with regard to an individual protein and the specificity of spontaneous renaturation of some soluble proteins following denaturation and scrambling of structure (*1*). The physical rules of refolding conform to the principles of thermodynamics and are consistent with the reduction of Gibbs free energy. In general terms, this implies a burying of hydrophobic groups within the folded molecular core of a globular protein along with the creation of ion-pairs and hydrogen bonds. The overall result is to minimize the surface area of the protein and reduce the structuring of water near nonpolar groups (*2*). The structure of proteins embedded within the lipid bilayer is also consistent with these principles. The ion-pairs and hydrogen-bonding groups of membrane-spanning proteins, along with bends and irregular turns of the protein backbone, should mainly be in areas of the protein exposed to the aqueous environment or hydrophilic portion of the bilayer, whereas the highly structured alpha-helix or beta-sheet (whose hydrophilic groups bind to one another) may be sequestered in the hydrophobic environment of the lipid (*3,4*). For example, relatively apolar groups of 25 amino acids may form transmembrane-spanning regions of membrane receptors, and algorithms have been developed to predict likely spanning regions from amino acid sequences deduced from cloned receptors (*3*). Application of these principles to recently cloned acetylcholine and Na^+ channels have revealed many clusters of amino acids potentially important for the gating and selective ion transport functions of these proteins (*5,6*). In the case of the voltage-dependent Na^+ channel (*6*), four internal repeated sequences of about 300 amino acids exist with a high degree of homology to one another. They are flanked by shorter stretches of nonhomologous residues. Because of this high degree of homology, it has been proposed that all these repeats arose by duplication of a common ancestor. They adopt similar secondary structures and appar-

orientations within the membrane. The homologous domains each contain several segments with significant alpha helical structure, and some of these may form the transmembrane pore of the channel.

What is particularly interesting about the structure of the voltage-dependent Na^+ channel is that it accomplishes within a single polypeptide what other Na^+ channels (for example acetylcholine-controlled Na^+ channels) accomplish with homologous or identical subunits (5). Evolutionary studies have great difficulty in differentiating convergence to a functionally useful structure and divergence from common ancestors. In studies of voltage- and ligand-gated Na^+ channels, the molecular taxonomist faces the problem of variation and similarity. For this reason, the recent cloning of the erythrocyte anion channel and the implications of its structure with regard to cloned ligand (GABA and glycine)-gated anion channels are discussed below.

Kinetics and the Binding Properties of Receptors

The same forces that control the conformations that proteins can assume are involved in the binding of ligands. The formation of receptor–ligand complexes is dependent on an overall negative free energy associated with the process. The free energy is related to the equilibrium constant by the relation $\Delta G = -RT \ln K$, and the free energy is composed of enthalpic and entropic factors that contribute to the overall reaction. Binding of ligands frequently has strong temperature dependences, and the optimum temperature for binding may bear no obvious relation to body temperature, where the drug has its biological effects. The reversible binding of ligands to receptor proteins involves noncovalent bonding, alterations in hydration of the ligand and the binding site, and changes in the conformational state of the receptor protein. Receptor proteins exist in several potential conformational states consistent with their functional properties; the binding of ligands may stabilize the protein in one of these states (7,8).

In the case of receptors, the agonist can interact with a resting state, capable of activation, resulting in a conformational change in the receptor molecule that causes the biological event, such as channel opening. Agonists also have the potential to interact with an inactivated, desensitized state or cause shifts in equilibrium to the side of the desensitized complex. In contrast, antagonists may prevent the binding of agonists to their site or act allosterically to block the event caused by the agonist. Since there may be multiple binding sites on the same receptor (as is the case for the GABA–benzodiazepine receptor complex), the actual functional state of the protein at any given time depends on which ligands occupy their sites. Multiple-affinity states of the receptor for the agonist also make it possible that the conformation of the receptor depends on the most recent occupancy of the binding sites on the protein; this results in long-lasting, metastable states and a "macromolecular memory." This phenomena is called hysteresis and indicates that a state of "equilbrium" may not give rise to equilibrium-binding curves (8). Depending on which drugs and agonists are added first and which states are the longest lived, varying conditions may be obtained during the finite period of a binding assay. As we will discuss below, enough evidence now exists for the GABA–benzodiazepine receptor complex to suggest that hysteresis and multiple-affinity states are important for this receptor complex. Thus, the literature related to the molecular biology of the inhibitory amino acid receptors can be seen as part of the vast literature related to the structure and function of allosteric proteins.

GABA/Benzodiazepine/Chloride Channel

The molecular biology of the receptor for the amino acid transmitter GABA is an area of re-

search in which much new information has been learned in the last 3 yr. Since 1985, the GABAergic system has been reviewed from the prospective of being a locus of minor tranquilizer action (*9,11*). Increasingly rigorous molecular evidence supporting this hypothesis has been generated, and the proteins that carry out these actions have been purified and cloned. Before discussing the receptor complex at a molecular level, it is important to discuss the findings leading up to these studies.

γ-Aminobutyric acid and glycine are two of the major inhibitory amino acid transmitters in brain. For over 30 yr, GABA has been described as a transmitter, and much work has been directed toward the involvement of GABA in various medical and neurological diseases (*12*). Glycine has been recognized as a component of proteins for close to 100 yr, and, in more recent times, as the major inhibitory transmitter in spinal cord (*13*). Both of these substances fulfill the major criteria for consideration as neurotransmitters; they are synthesized and stored in a limited number of neurons in the central nervous system, they undergo electrically and chemically induced release, carrier-mediated uptake and mechanisms for inactivation have been demonstrated in brain, they mimic the actions of endogenously released inhibitory substances, and receptors for these substances have been found in brain. The molecular properties of these receptors are the subject of this review.

Molecular Topology of the Complex Deduced from Binding Studies

GABA Site

To review the entire literature of GABA receptors would require extensive consideration of both vertebrate and invertebrate receptor species and is outside the scope of this review. With the cloning of the mamalian chloride channel-linked GABA receptor, it should be possible to examine, using molecular genetic techniques, the phylogenetic linkages of GABA receptors in a number of species. However, since this data is not yet available, only the chloride channel-linked GABA receptors, called $GABA_A$ receptors, will be considered here. It is known that a second type of GABA receptor ($GABA_B$) exists in mammalian species (*14*), and its actions are mediated through a guanine nucleotide regulatory protein (G protein); these effects include modulation of adenylate cyclase and direct effects on ion channel opening (*15*). Many of the effects seen in invertebrates may be related to GABA's action at receptors, which appear to be related to $GABA_B$ receptors (*16*), whereas others may be related to effects on chloride conductance.

Sodium-independent binding of [^{3}H]GABA to synaptic membranes prepared form rat brain was one of the earliest biochemical studies of the $GABA_A$ receptor in brain. It was then learned that freeze-thawing and treatment of the membranes with low concentrations of nonionic detergents increased the affinity of GABA receptors for GABA and unmasked a high-affinity site (or state) for GABA (*18*). After almost 10 yr, it is still not clear what the relationship is between the high- and low-affinity states on the GABA receptor; it is also not known what the relative binding to $GABA_A$ and $GABA_B$ is after such harsh membrane treatment. Most of the biochemical studies of of GABA receptor binding have characterized the high-affinity binding site, whereas the electrophysiologists study a much lower-affinity state. Binding to membranes that are dramatically altered in their structure by detergents and freeze–thaw cycles clearly does not reflect physiological function; however, much of the pharmacology deduced from these studies and electrophysiology is quite similar.

Binding to the high-affinity state of the GABA receptor is stereospecific for the antagonist bi-

cuculline, the (+) form is almost 100 times more potent in inhibiting GABA binding than the (–) (*19*). Through the use of [^{3}H]-(+)-bicuculline, a method for assessing binding to the high- and lower-affinity state of the GABA receptor was developed (*20,21*). This assay depended on the use of freshly prepared (rather than frozen) membrane fractions, and the inclusion of thiocynate in the incubation media [thiocynate had been shown to eliminate the high-affinity component of GABA binding (*22*)].

Because of the instability of bicuculline derivatives, this assay is not routinely used, but along with the GABA shift in benzodiazepine receptor affinity, it is the best currently available method to study the lower-affinity states of the GABA receptor. A shift in GABA affinity from the lower- to higher-affinity states has been found in conditions in which electrophysiological subsensitivity to GABA has been demonstrated [chronic diazepam, *see* below and ref. (*23*)]. This has led to the hypothesis that the high-affinity state of the GABA receptor may represent a functionally desensitized state, but a highly favorable protein conformation (*9*).

Rigid analogs of GABA have been proven to be important tools in the study of the requirements of GABA receptors measured electrophysiologically (a low-affinity state), by direct binding assay (high-affinity state) and indirectly through increases in benzodiazepine receptor binding (low-affinity). Some general conclusions arise from examining their structures (Fig. 1). All compounds with effects on GABA receptors have zwitterionic structures, with the charged groups spaced appropriately from one another (*24*). The carboxyl groups of GABA can be substituted with other groups, such as sulfonic acids in 4-aminopropanesulfonic acid (4-APS) (*25*) or isoxazolol groups (muscimol is an example) (*26*) without the loss of activity, and rigid rotational analogs possessing great potency can be prepared (*24*). Extensive delocalization of the negative charge in some muscimol derivatives does not prevent the compounds from binding to the receptor, but may interfere with the agonist properties of these compounds (*26*). Extensive evaluation of these compounds through the different in vitro binding assays described above has revealed subtle differences in each class of compound as potential agonists at the GABA receptor. One derivative, 4,5,6,7-tetrahydroisoxazolo[5,4-c]-pyridin-3-ol (THIP) has been classified as a partial agonist based upon its weak ability to enhance benzodiazepine binding compared to muscimol (*27*). In electrophysiological assays using extracellular recording, it appears to be a full agonist, and taken together these findings have led investigators to postulate different GABA receptor-gated chloride channels, some benzodiazepine linked and some independent [discussed in ref. (*28*)]. Although this is a possibility, a simpler explanation for differences in the predicted properties of these molecules exists. The residues on the GABA receptor available for interaction with the charged groups (and rigid groups that induce slight structural differences in these molecules) of the GABA analogs are different because of the stability of particular conformations of the receptor obtained when tissue is prepared by various methods and incubated in different ionic conditions. These nonphysiological conditions are then compared to conditions for recording from intact cells. Some resolution of these differences has been obtained with voltage-clamp studies of isolated patches (*see* below). Further clarification will come when cloned GABA receptor subunits are expressed in mammalian cells.

A current picture of the possible activation states of the GABA receptor complex is shown in Fig. 2. This model shows some of the occupancy states of the $GABA_A$ receptor and its two putative regulatory sites, which are described below.

Benzodiazepine Site

In spite of changing attitudes concerning the use of minor tranquilizers since the early period

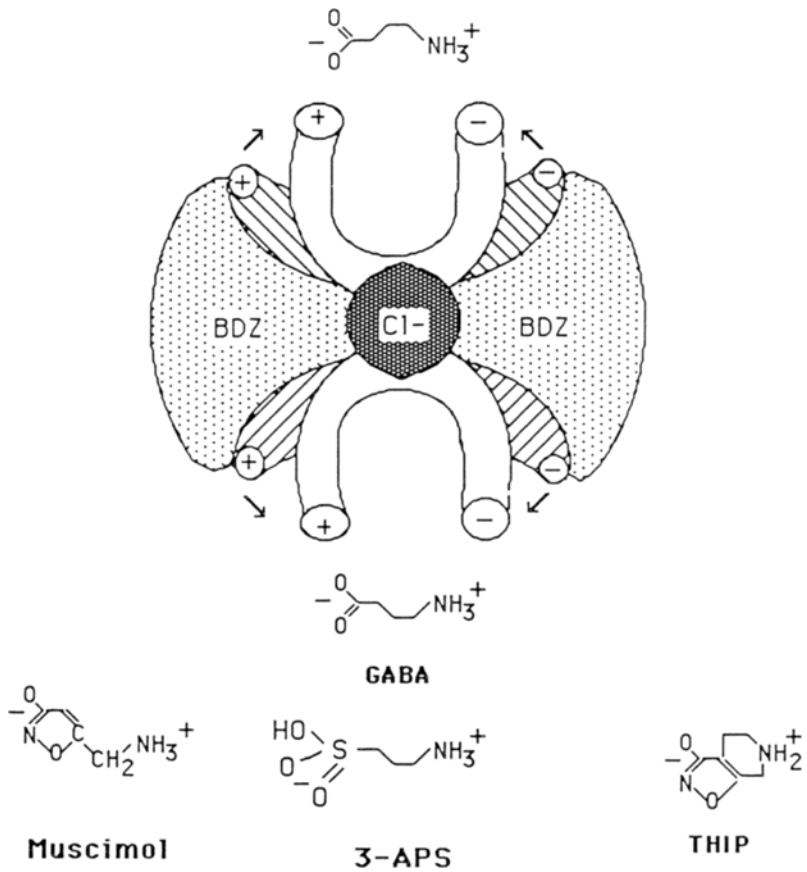

Fig. 1. Diagramatic representation of the interaction of GABA agonists with the GABA receptor complex. Compounds that interact with the complex, such as GABA, muscimol, and 4-aminopropane sulfonic acid (4-APS) have zwitterionic structures. Binding of these agonists (probably two per event) to form salt bridges with the receptor results in a conformational change of the inactive receptor (hatched area) to an active state (open area), which causes the opening of the chloride ionophore. Benzodiazepines modulate the action of GABA at closely aligned binding sites.

of their introduction in the 1960s, various analogs of the benzodiazepines continue to be one of the most widely used classes of drugs in the world (*29*). The pharmacogical properties of these compounds have been extensively studied and reviewed else-where (*9–11,30*), and in this article only chemical aspects of the structure of these drugs as related to the topology of the benzodiazepine receptor will be considered.

Early electrophysiological studies of the actions of the benzodiazepines indicated that one of the major actions of the benzodiazepines was to enhance GABA-ergic transmission in the nervous system (*31,32*). By the mid-1970s, a number of other transmitter systems had been implicated indirectly (and in some cases incorrectly) in different aspects of the behavioral actions of these drugs [*see* refs. (*31,32*) for review]; although the molecular pharmacological (and now molecular biological) evidence obtained from that period to the present indicates that the site of action of these drugs is on the GABA receptor complex itself, it remains for the next generation of neuroscientists to investigate how

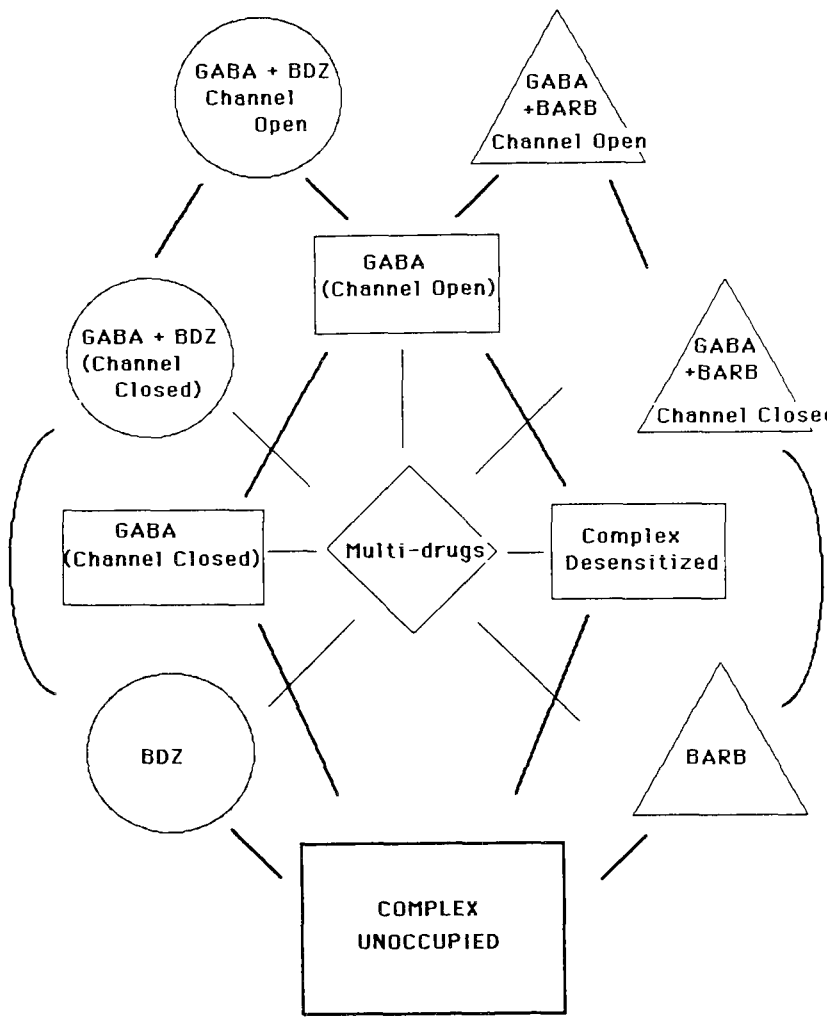

Fig. 2. Schematic diagram of the complexity of the relationships that can be obtained when various drugs occupy the GABA receptor along with GABA itself. Each of these relationships can be represented by a separate equilibrium equation, resulting in a rather complicated overall series of rate equations to represent transitions from one state to another. Multidrug states in which both barbiturates and benodiazepines are present provide a situation that is even more difficult to accurately model kinetically.

the GABA system can influence other transmitter systems and control the behavioral output of different areas of the brain.

Studies on the mechanistic aspects of interaction of benzodiazepines with brain depended upon the demonstration of a high-affinity binding site (which we call a receptor) for the benzodiazepine diazepam (*33,34*). Binding to these sites is rapid, reversible, saturable, and stereospecific; it is also possible to show correlation between binding to this site by a chemically diverse group of compounds and their activity in a number of behavioral and clinical paradigms characteristic of the actions of the benzodiazepines (*31*). Binding to this site is also predictive of the in vivo properties of such com-

pounds, and binding assays are now widely used to develop new pharmaceuticals.

In 1978, it became clear that GABA and related analogs could interact at a low affinity (1–5 μ*M*) GABA binding site to enhance the binding of benzodiazepines at the central-type benzodiazepine binding site (*25*). This enhancement was caused by an increase in the affinity of the benzodiazepine binding site related to the occupancy of the GABA receptor by an agonist. The data were interpreted to mean that both the GABA and benzodiazpine sites were allosterically linked in the membrane and most likely part of the same complex of proteins. For a series of analogs, the ability to enhance the binding of the benzodiazepines and the ability to inhibit high-affinity GABA binding were directly correlated; however, the absolute potency of these activities was quite different (by two orders of magnitude). As mentioned above, the various measures of GABA potency in different tests correlate fairly well. The issue of what constitutes a partial GABA-ergic agonist was discussed above and in the section on patch-clamp studies. The current interpretation of the data is that several potential affinity states of the GABA receptor exist, and it is one of the lower-affinity states that is involved in the enhancement of benzodiazepine binding and the electrophysiological events. Under conditions in which the electrophysiological responses to GABA are diminished (desensitized), the ability of GABA to enhance the binding of the benzodiazepines is also diminished (*36*). The mechanistic aspects of desensitization and the shifts in GABA affinity states are not understood, and these studies will be enhanced by the increased amounts of receptor subunits that can be prepared using recombinant technology.

Agonist and Antagonist Domains of Benzodiazepine Receptors

Soon after the discovery of the benzodiazepine receptor, a series of compounds related to tryptophan were isolated from human urine based on their ability to inhibit benzodiazepine receptor binding (*37,38*). When their structures were determined, it became clear that they had been formed during the isolation precedure; methyl, ethyl, or propyl derivatives could be prepared by tissue extraction with the respective alcohol. Synthesis of a number of analogs of the basic β-carboline structure allowed a number of investigators to test their activity in behavioral paradigms. It was immediately realized that these compounds could antagonize the activity of diazepam (*39*) and possessed their own intrinsic activity opposite to the benzodiazepines. These activities include proconflict, convulsant, anxiogenic, and insomniac properties (*9*). Such activities have led to the naming of these compounds as inverse agonists (*40*).

A biochemical test for these inverse agonist properties emphasizes their interactions with the GABA-ergic system. In contrast to the benzodiazepines, which show increased binding in the presence of GABA, the β-carbolines show a decrease in their affinity for the benzodiazepine site in the presence of GABA agonists.

A third set of compounds shows no shift in its affinity in the presence of GABA; these compounds, particularly Ro15-1788, are antagonists of both benzodiazepine and β-carboline binding (*41,43*). They also antagonize the behavioral actions of benzodiazepine agonists and inverse agonists, but seem to be relatively inactive by themselves. A final set of compounds, which includes the pyrazolopyridazines (CGS-9896), are compounds that have a GABA shift that is to increased affinity, but with a magnitude smaller than benzodiazepines. These compounds may be partial agonists and are potentially the most interesting compounds because of their lower potential for the development of tolerance (*9*). A picture of the overlapping specificities of the active site is seen in Fig. 3.

The overall conclusions to be drawn from the examiniation of these drugs with a pharmacol-

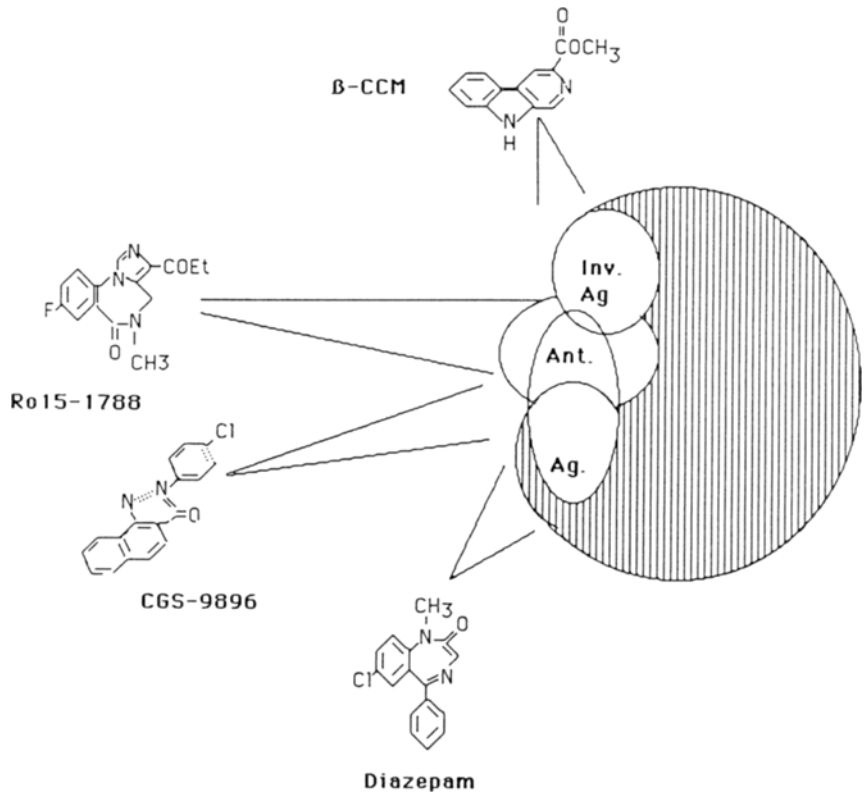

Fig. 3. Ligand interactions at the benzodiazepine receptor may be classified as agonist (Ag.), inverse agonist (Inv. Ag.), or antagonist (Ant.); the binding sites for these compounds are distinct but overlapping sites on the GABA–benzodiazepine complex. Agonists, such as diazepam, are anxiolytic, sedative, muscle relaxant, and anticonvulsant, whereas inverse agonists, such as β-carbolines (β-CCM), have anxiogenic, insomniac, and convulsant properties. Antagonists, like Ro 15-1788, have little intrinsic activity of their own, but block the other series. CGS-9896 may be a partial agonist.

ogical spectrum of activities ranging from the sedative to the convulsant is that this "receptor" is not a receptor in the classical sense. There is probably not a transmitter (endogenous benzodiazepine ligand) that interacts normally at the benzodiazepine site, and the benzodiazepine receptor ligands do not synergize with, substitute for, or block the actions of such a transmitter. The actions of these benzodiazepines and related drugs are primarily modulatory on the GABA system. This can account for their opposite interactions and diverse spectrum of activity. In regions of the brain where GABA levels are high and the GABA neurons are quite active, the benzodiazepines would have relatively lower activity. This is because their action is essentially to make GABA a more potent transmitter, and since GABA activity is proceeding at a high rate, their activity is minimized. In regions where there is little GABA, the effects of the benzodiazepines are more profound Because the benzodiazepines do enhance the activity of small amounts of GABA and make it more likely that GABA will exert an action at the receptor. In contrast, the β-carboline inverse agonists might be expected to have blocking activities in a manner opposite the the benzodiazepines; in areas of high GABA-ergic activity, thier ability to decrease the efficacy of GABA might be more pronounced than in areas where

there is little active GABA transmission.

Antagonists, such as Ro15-1788, possess little pharmacological activity of their own. They do not seem to be antagonizing a major endogenous transmitter system, and the activity that is present is slightly benzodiazepine-like in drug-naive animals; in animals chronically treated with benzodiazepines, Ro15-1788 induces withdrawal reactions that are quite prominent and are probably related to the development of GABA-ergic subsensitivity (*44*). Blocking the benzodiazepine receptor under these conditions would result in hyperexcitability related to the removal of a tonic pro-GABA chronic benzodiazepine receptor occupancy.

An endogenous "endocoid" has been isolated from brain that seems to also modulate the actions of GABA. This compound is a fairly large peptide and may subserve the function of a modulator of the diazepam portion of the GABA–receptor complex (*45*). Its pharmacological action has been described as opposite to that of GABA, and it is thought to modulate the activity of the GABA–receptor complex. Diazepam might inhibit its action. This and several related proteins have been cloned, and mRNAs coding for them have been found in a number of tissues (*46–38*). The levels in peripheral tissues have raised the question (*48*) whether this family of proteins are transmitters, as is generally accepted. They may actually be subunits of known proteins in the periphery, and a regulatory role analogous to that of the regulatory units of protein kinases may underlie the action of these proteins in brain.

Photoaffinity Labeling of the Benzodiazepine Receptor

Receptors frequently have a very high affinity for the drugs with which they interact. In the usual case, the binding of drugs is reversible and effective study of the receptor requires the ability to rapidly separate free and bound drug. If the drug contains a potentially reactive group, then it may be possible to form a covalent bond to the receptor protein while it is near the binding site. Reversibly bound ligands of even the highest affinity will dissociate during chromatography or electrophoresis under denaturing conditions and are not suitable for identification of individual protein species. Covalently linked ligands to detect the protein allow the use of time-requiring or denaturing conditions. A recent review of photoaffinity labels as pharmacological tools reviews the advantages of different reactive groups that can be used for this purpose (*49*).

The first compound prepared that was shown to form a covalent bond to the benzodiazepine receptor was a derivative of flurazepam containing an isothiocyanyl group as the reactive group (*50*). This compound was called irazepine [1-(2-isothiocyanatoethyl)-7-chloro-1,3-dihydro-5-(2-fluorophenyl)-2H-1,4-benzodiazepine-2-one]. It was a noncompetitive inhibitor of [^{3}H]diazepam binding and appeared to be irreversible. The nature of its reactions with model compounds indicated that irazepine probably interacted with a lysine or cysteine near the receptor's active site; the actual fragment of the receptor was not isolated. A related bromoacetyl derivative, called kenazepine, had similar effects (*51*). These compounds were capable of distinguishing apparent benzodiazepine receptor subtypes and led to a primitive description of apparent receptor domains (*52*). The major limitation of these compounds was a lack of a radioactive analog that would have permitted radiolabeling and identification of the benzodiazepine receptor protein; these derivatives were not developed because soon after the synthesis of the alkylating benzodiazpines, the photolabeling properties of nitro-containing benzodiazepines were discovered.

Among the large number of compounds developed by Hoffmann-LaRoche as potentially useful drugs, a highly active series of derivatives containing a 7-nitro group was synthesized and marketed as hypnotics (flunitrazepam, Rohypnol) or anticonvulsants (clonazepam, clonopin) (53). These compounds contain

a nitro group rather than a halogen at position 7, and the nitro groups are capable of forming a number of resonance structures with the heterocyclic rings. These compounds possess strong absorbances in the near UV portion of the spectrum and in the presence of UV-light can form highly reactive free radicals. When these compounds are localized in a water-free hydrophobic part of the receptor, the free radicals are capable of forming crosslinks to the benzodiazepine receptor.

Using [^{3}H]flunitrazepam, the usual reversible binding could be transformed into a covalent bond by exposure to UV light (*54*). Since the formation of this bond depended on occupancy of the benzodiazepine receptor, the photolabeling of the receptor was blocked by nonradiolabled benzodiazepines. The potency of these compounds as blockers of photolabeling was directly correlated with their potency as reversible ligands. Photolabeling with flunitrazepam could only be demonstrated with the central-type benzodizepine receptor; no specific photolabeling was obtained in kidney or C_6 glioma cells, both of which possess binding sites for the benzodiazepines with high affinity for Ro5-4864 [ref. (*55*) *see* below]. Ro5-4864 is identical to diazepam, except that it possesses a *p*-chloro substitution on the phenyl ring, and this single substitution renders it very inactive at central-type benzodiazepine receptors (*55*).

Photoaffinity Labeling Reveals Localization of Benzodiazepine Receptors

The covalent incorporation of radiolabeled flunitrazepam into benzodiazepine receptors, using the techniques described above, has allowed the expansion of autoradiographic localization studies from the light (*56–58*) to the electron microscopic level (*59*). These elegant electron microscopic studies provided evidence for the neuronal localization of benzodiazepine receptors. Immunocytochemical staining for glutamic acid dehydrogenase, the committed step in GABA synthesis, frequently colocalized with the radiolabeled flunitrazepam. However, such studies do not provide evidence for a pre- or postsynaptic localization for benzodiazepine receptors because the electron microscopic autoradiography does not provide sufficient resolution. Further studies with monoclonal antibodies (*see* below) have clarified this issue.

The ability to incorporate radioactively labeled flunitrazepam into the benzodiazepine receptor has allowed the examination of labeled receptor by electrophoresis in the presence of sodium dodecyl sulfate (SDS). All researchers have demonstrated the presence of a major band from rat brain with mol wt of approximately 50,000 (*54,55*). An additional band of mol wt 55,000 has been shown to be prominent in hippocampus and striatum (*60,61*) and appears with minor bands early in hippocampal development (*62*). The 50,000 protein has been termed the BZ_1 receptor and the 55,000 protein the BZ_2 receptor. Further minor bands with sizes of 53,000 and 59,000 have also been demonstrated (*61*).

The interpretation of these studies in terms of receptor subtypes will have to undergo revision based upon the molecular biological and antibody studies described below. What has emerged from those studies is that the 50,000 protein is now known as the α subunit and a 55,000 protein is known as the β subunit of the GABA–benzodiazepine receptor complex; they both exist in all regions, as seen with monoclonal antibodies directed against the purified GABA–benzodiazepine receptor complex. Their amino acid composition is different. It is not entirely clear what the relationship is of the 55,000 protein identified by photolabeling and the protein identified by the antibodies (and cloned); the 55,000 protein may represent regionally variable labeling of the β subunit or be a separate gene product.

In some photolabeling studies, no attempt was made to inhibit proteolysis. Endogenous proteases are apparently present in membrane fractions and could contribute to the number of

bands seen on SDS gels. (*63*). Another hypothesis is that heterogeneity could result from region-specific processing of precursor forms of GABA receptor subunits. This may result in slightly different carbohydrate side chains or incomplete removal of (leader ?) sequences in pre- versus postsynaptically localized receptors. In the case of at least one membrane protein, Na^+,K^+ATPase (*64*), alternate splicing of the genes leads to altered protein structure. Altered processing could also account for the changes in molecular weight seen during development. Along with the size differences, neonatal animals show a much larger effect of GABA on benzodiazepine binding then do adult animals (*65*), indicating that the structural changes may be reflected in functional differences. Thus, the multiple forms of the receptor may represent "isoreceptors" (genetically distinct forms of receptors). With more sophisticated probes, it is almost certain that such isoreceptors may emerge as important in investigations of the etiology of psychiatric disorders in people.

In pharmacological studies, it has been claimed that the BZ_1 receptor has high affinity for a pyrazolopyridazine (CL 218,872). This compound is of interest because it was the first compound that did not interact with the binding of radioactive benzodiazepines with a Hill coefficient of 1, which would indicate unity in the binding site; this receptor heterogeneity was seen prominently in hippocampus (*66,67*). In contrast, this compound did not distinguish apparent benzodiazepine subtypes in the cerebellum that contain only BZ_1 receptors; cerebral cortex also contains mostly BZ_1 receptors. Several unsubstantiated claims were made for the pharmacological properties of the pyrazolopyridazines; the most important of these is that the pyrazolopyridazines possess potent anticonflict and anticonvulsant activity with little sedation (*68*), which were thought to be mediated through BZ_1 receptors. More recent appraisal of these compounds indicates that because they do possess activity at BZ_2 receptors and because the receptor-specific activities are not very different in potency, a clear in vivo effect mediated by a benzodiazepine receptor subtype might be hard to distinguish. Under certain circumstances, they possess proconflict activity (*69*). A smaller GABA shift (*70*) and a smaller photoshift (*71*) than benzodiazepines has also been found for these compounds. The final conclusion is that they may be more appropriately classified as partial benzodiazepine agonists (*72*).

The ability of GABA to differentially alter the binding of benzodiazepine agonists and antagonists means that the loci to which the agonists and antagonists bind cannot be identical. In the presence of GABA, the affinity increase in flunitrazepam binding to the benzodiazepine receptor is related to a change in the entropic parameter of benzodiazepine binding when the study is carried out at 4°C. At 37°C, an additional change in the enthalpic term also occurs (*73*).

The separate domains of binding of benzodiazepine agonists and antagonists can also be defined by examining the parameters of ligand binding following photolabeling. During the initial studies of photolabeling, it was discovered that photolabeling with flunitrazepam could cause covalent crosslinks to the receptor, as described above. However, when the total number of binding sites on the membrane was compared to the number of sites being photolabeled, it was discovered that only about 25–40% of the sites were labeled. In addition, the remaining sites were apparently inactivated when reversible binding of benzodiazepines was studied in tissue that has been previously photolabeled. In contrast to the drastic diminution of the agonist binding site, the antagonist binding parameters (studied by Ro15-1788) or inverse agonist binding (studied by β-carboline binding) were relatively unaffected by photolabeling (*71,74*). When benzodiazepine agonists were used to displace the binding of antagonists and inverse agonists the displacement curves

were shifted to lower potency and were more shallow. These shallow curves indicated heterogeneity in the sites to which the benzodiazepines bound; the photoshift was sensitive to full and partial benzodiazepine agonists; unlike the GABA shift, it was not sensitive to the properties of antagonists or inverse agonists. The pyrazolopyridazines by this test were partial agonists.

A number of interpretations are possible for the greater inactivation than labeling of sites with flunitrazepam. Among these interpretations, the most interesting is that there is site-to-site interactions between binding sites for benzodiazepines. This is also consistent with the current allosteric models for receptor function and the presence of complex binding curves for GABA-ergic agonists. The complex pattern of interactions should be clarified by the definition of the stoichiometry of the complex following its cloning. The electrophysiological effects of GABA require the binding of two molecules to initiate the physiological response. The effects of the benzodiazepines are not to increase the affinity of GABA for receptor, but to increase the probability that when such an event occurs, opening of a chloride channel will occur. By viewing the system in terms of GABA, a greater appreciation of the interactions between the sites in the complex may be obtained. As mentioned above, the stoichiometry of the GABA and benzodiazepine sites has not been defined, but appears to be more complicated that one-to-one, and a full description will require the purification of these sites and their reconstitution.

Membrane-bound, photolabeled benzodiazepine receptors are susceptable to degradation by an endogenous trypsin-like activity present in well-washed membrane fractions (*63*). The presence of this activity suggested that controlled, proteolytic degradation of the membranes might release small fragments of the receptor that would be suitable for further purification. Since [^{3}H]flunitrazepam can be incorporated into the receptor before proteolysis, it would provide a convenient way to monitor the fragments released from the receptor during purification. A procedure (Klotz et al., unpublished) was developed to release proteolytic fragments of the photolabeled receptor and to purify such small molecular weight hydrophilic fragments released from the membrane. This method used sequential filtration to remove substances with molecular weight greater than 10,000, gel filtration, and several reverse-phase, high-performance liquid chromatography (HPLC) runs. The method was suitable for the preparation of several micrograms of labeled receptor fragments, and a single major limit peptide with no further proteolytically susceptable sites was obtained by controlled proteolysis with a mixture of trypsin and chymotrypsin.

Amino acid sequence analysis of this peptide was carried out by automated Edman degradation, identifing phenylthiohydantoin-derivatized amino acids by HPLC on an IBM cyano column. The analyzed sample was greater than 95% a single peptide by end-group analysis and contained the entire radioactivity of the major isolated fraction. The sequencing was performed on two entire, independently prepared samples from separately collected brains, and a likely sequence deduced is: Pro-Ala-Pro-Ala-Thr-Thr-Phe(F)-?Lys(K).

X may be the amino acid to which flunitrazepam is linked. A related sequence has now been found in the cloned 50,000 α subunit of the benzodiazepine receptor, but not in the 55,000 protein (Barnard, personal communication to Tallman).

Homologous Amino Acid Sequences in Other Proteins

The sequence PAPA is found in a number of vertebrate proteins that have been recently isolated and sequenced. These include myosin light chain, H_1 histone, troponin, and immuno-

globulin heavy chain. Taking a slightly longer view, the sequence PAPATTFAH has been found in spinach tobacco and maize ATPases. The sequence PAPAUTF is found in myosin β chain ATPase, and a related sequence, PSPAT-TFAH, is characteristic of the β chain of a protomotive ATPase from *Escherichia coli* (*75*). The approach described above for the purification of receptor fragments is generally applicable to any receptor system in which a radiolabeled covalent ligand can be linked to the binding site. Using this approach, a partial sequence that has served to identify potentially related proteins was obtained. This sequence is found in a number of ATPases and most importantly is in a region of the ATPase that is involved in the binding of adenine nucleotides. This may explain why the purines possess activity in displacing benzodiazepine binding and were at one time thought to be potential endogenous ligands for the receptor (*31*).

We cannot deduce from this structure alone where it is localized in the receptor molecule; however, an interesting picture of the possible form of the α subunit of the GABA–receptor complex may be made localizing the sequence on the outer side to the membrane (Fig. 4). This is consistent with the ability of drugs to rapidly bind to the benzodiazepine receptor, which is presumably localized on the cell surface.

Picrotoxin/Chloride Channel

The binding of benzodiazepines to the benzodiazepine receptor is enhanced by a number of anions, including iodide, bromide, nitrate, thiocyanate, and chloride (*76*). The selectivity of the effective anions correlated with their selectivity for penetrating the activated postsynaptic membrane of cat motorneurons and was interpreted to indicate the proximity of the anion site to the chloride gating function. Almost all the drugs that interact at the set of sites near the chloride channel show some modulation of their binding effects with anions, and this anion binding site seems to be quite important for these functional interactions.

Picrotoxin is a sesquiterpene produced by plants of the Menispermaceae family and has been known as a convulsant for many years; its mechanism of action as determined from electrophysiological studies is as a noncompetitive GABA antagonist. It is different from bicuculline in that it does not block the GABA receptor directly, but seems to block the channel that mediates the action of GABA (*77,78*). Analogs of picrotoxin were initially used as radiolabels for this "channel-linked" site; however, nonspecific binding made this ligand unsuitable for careful investigation of allosteric effects. Binding to the picrotoxin site was inhibited by a number of compounds with inhibitory and excitatory effects on the central nervous system. One of these classes of compounds is the barbiturates that interact with the picrotoxin site roughly in the order of their potency as hypnotics and anesthetics (*79*). A number of the barbiturates and related compounds are also capable of enhancing the binding of benzodiazepine agonists to the benzodiazepine receptor, which may indicate allosteric interactions between these two sites (*77–80*). An additional set of compounds, called "cage convulsants," also interact with this channel-related site, and one of these compounds, *t*–butylbicyclophosphorothionate, (TBPS) has been important in defining more clearly drugs that are capable of interacting with this channel-related site (*80*). Picrotoxinin, TBPS, and some barbiturates all seem to interact with both a common portion of the GABA receptor-associated chloride channel and a unique site for each chemical species. Extensive study of the interactions of each compound with its binding site and their respective interactions with GABA and benzodiazepine binding sites under a rather large set of temperatures, tissue conditions, and ionic states has lead to hours of amusement for pharmacologists interested in complex interactive effects.

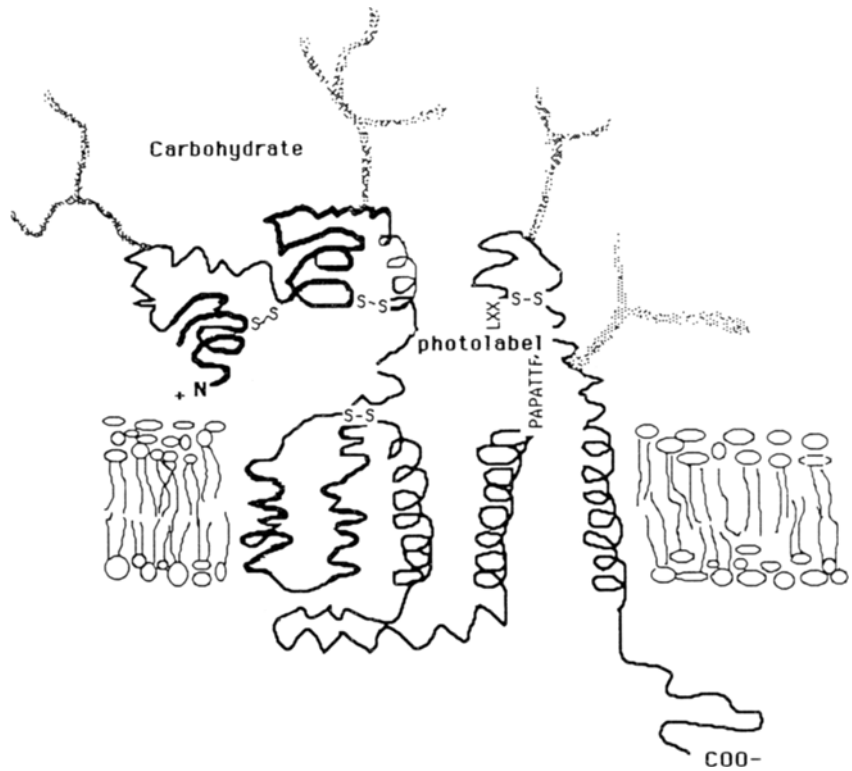

Fig. 4. The benzodiazepine receptor is depicted as an integral membrane protein. The receptor has been shown to be a glycoprotein by glycosidase studies, with carbohydrate making up 10–20% of the apparent weight. The photolabeled fragment is included on the outside of the membrane, where it may participate in forming the active site for benzodiazepines.

Molecular modeling of the picrotoxinin site has provided some insights into the steric requirements of compounds that work at this site and the molecular properties of the putative chloride channel (*81*). Using various analogs of picrotoxinin and gamma-butyrolactone [a chemical series that contains both convulsant and anticonvulsant compounds (*82*)], three-dimensional models of their overlapping receptor sites were constructed with the aid of a molecular graphics computer system. The differences between active and inactive compounds and those possessing convulsant and anticonvulsant activity were calculated. One of the assumptions of this study has been that these compounds bind to a site that overlaps the chloride channel itself. The relative abundance of the chloride overlapping conformations were calculated and the unique portion of the convulsants is that volume occupied by the β-alkyl group substitutions on the butyrolactones; this structure is found both in the butyrolactones and picrotoxinin molecules (Fig. 5). It is possible to orient the presumptive chloride channel and chloride ion such that they could overlap with these structures and they could at least partly block chloride entry. From this sort of modeling, predictions can be made about the pharmacological design and relative properties of potential anticonvulsants. Direct testing of molecular modeling of this sort through protein–drug interactions will await the production of large amounts of receptor protein and X-ray crystallographic studies of the active site. It

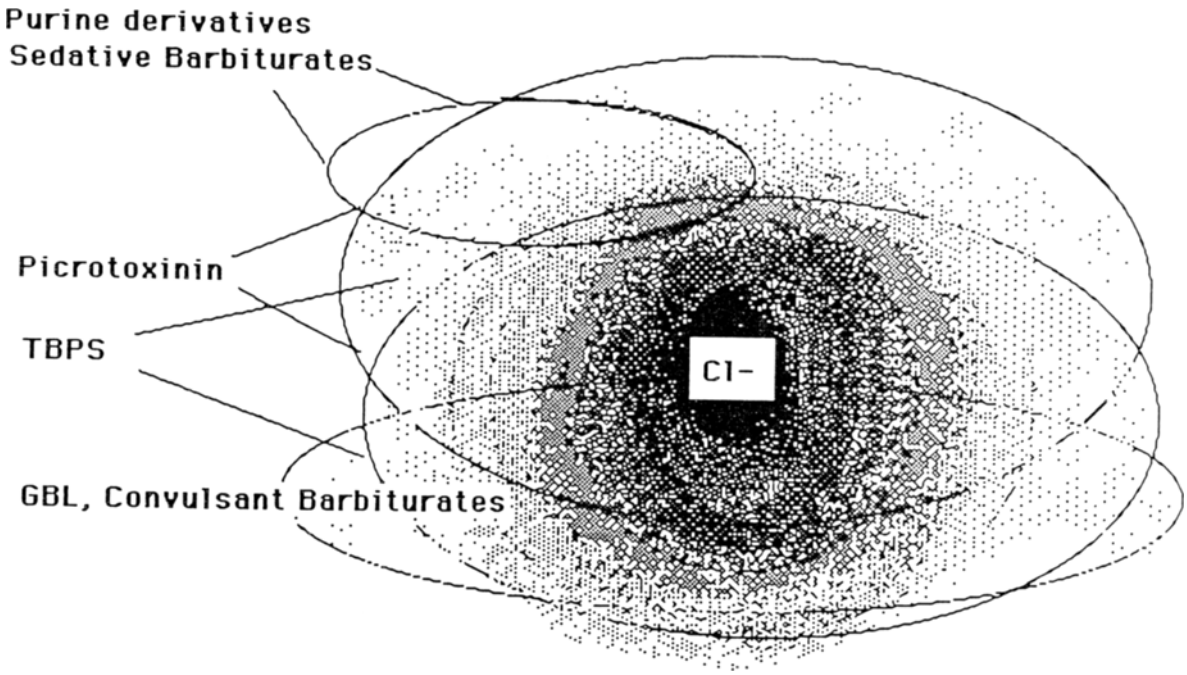

Fig. 5. Schematic diagram of the chloride ionophore portion of the GABA receptor complex and associated domains for drugs interacting at these sites. Drugs that affect the chloride channel bind to different but overlapping sites. Picrotoxinin and the cage convulsants (TBPS) interact with a channel-related site, and binding to this site is inhibited by sedative barbiturates. γ–Butyrolactone (GBL) and convulsant barbiturates bind at another channel-related site, which overlaps with the picrotoxinin site.

is not yet clear how the picrotoxin sites overlap with the barbiturate and TBPS sites, but it should be noted that a series of barbiturates with stereochemically determined convulsant and anesthetic properties exist that could be analyzed in the same way (*78,82*). A picture of the overlapping domains of the chloride channel is shown, with the putative channel-blocking groups highlighted (Fig. 5).

Structure and Function Deduced from Patch Fluctuation Analysis

Extensive analysis of the responses of neurons to GABA and the drugs described above have been carried out using sophisticated electrophysiological techniques. Using statistical techniques developed for noise analysis in communications networks, analysis of voltage fluctuations induced in spinal neurons in culture by GABA and related compounds has allowed quantitative description of the elementry processes associated with the stimulation of the GABA receptor. Without entering into the technical details of these analyses, measurement can be made of the conductance (γ, channel opening) and the duration of opening (τ, mean channel open time) (*83*). Since there is little voltage dependence of either parameter, they are almost entirely controlled both by the occupancy of the GABA receptor and its related allosteric sites and by the relaxation of the activated receptor complex from its various states (*84*).

Subjected to this analysis, several interesting aspects of the actions of the GABA-agonists

emerge. In rat spinal cord cultures, all GABA analogs activate membrane channels whose conductance is similar to that activated by GABA itself. However, the mean lifetimes of analog-induced channels may be quite different from GABA (mean channel open time of 29 ms). For example, the debate about THIP was discussed earlier along (comparing binding properties with a less sophisticated analysis of the electrophysiological data); subjected to fluctuation analysis, it becomes clear that the mean channel open time induced by THIP is 11 ms (*85*). If the actions of THIP mediated through its interaction with the GABA binding site are considered, the kinetics of the GABA receptor-coupled anionic channel opening are different from GABA, and this difference in pharmacology may depend on the structure of the agonist tested. In contrast to THIP, muscimol activates a chloride conductance with a mean duration of 65 ms, almost six times as long. To compare both these compounds and call them both full agonists is clearly a gross simplification of a very complicated kinetic situation; it is the binding studies and simple interpretation of electrophysiological data that are deficient here because they are only hinting at differences in the structures and their physiological consequences. Since THIP possesses a very small GABA-shift of benzodiazepine binding and blocks GABA-activation of benzodiazepine binding, it fits the classic biochemical description of a partial agonist; since it activated a chloride conductance, it is an agonist, and, without the analysis above, data would indicate that it operates the same conductance as GABA ("full agonist"). Only when subjected to the above analysis does the real picture of its differences from GABA emerge. It remains for another generation of kineticists to study the low-affinity GABA sites when enough protein is available to use fluorescent techniques to evaluate real-time binding equivalent to the electrophysiological assay as opposed to steady-state conditions.

Fluctuation analysis has also been carried out on the response of GABA-receptors to a combination of GABA and the benzodiazepines (*85*). The GABA responses in the presence of benzodiazepines are associated with an identical conductance to those induced by GABA alone, and small differences seen are not sufficient to account for the increased membrane current response. However, diazepam seems to increase the frequency of the channel opening in response to GABA and only slightly alters the mean duration of channel opening. Thus, the efficacy of GABA in causing channel opening is increased (*86*).

The anesthetic barbiturates are also capable of enhancing the actions of GABA in a variety of preparations by increasing the mean channel open time and decreasing slightly the frequency of channel opening. Since there are some effects of pentobarbital on GABA binding to its receptor, these may also contribute to the overall effect (*85*). Such effects are consistent with the hypothesis drawn above that the site of action of the anesthetic barbiturates is close to the chloride channel itself.

Patch-clamp studies have revealed additional aspects of GABA-receptor channels in spinal neurons and adrenal chromaffin cells (*87–89*). Fast application of GABA to a whole adrenal chromaffin cell in voltage clamp revealed a dose–response range to GABA in the low μM range and rapid desensitization at 20 μM (*88*). Extrapolation of log dose curves versus peak response allowed the calculation of a Hill coefficient of 2 for this response and indicates that two molecules must bind per event. The GABA-activated single channel currents displayed at least three different conductance levels in chromaffin cells (*88*), similar to glycine responses in spinal cells (*87*). However, glycine did not open channels in chromaffin cells. Thus, the data from patch-clamp studies indicate a more complicated gating behavior than previously anticipated from the fluctuation analysis studies. Channel closings may show complicated ki-

netic behavior involving both channel reopening during a single occupancy of the receptor (Nachschlag phenomenon) and rapid (ms) desensitization related to the presence of agonist (*89*). In the presence of diazepam, the GABA-activated currents were potentiated by a factor of two, and the time course of desensitization appeared to be somewhat faster (*87*). Clearly, the biochemical studies are orders of magnitude away from studying these phenomena kinetically at the present time; with large amounts of protein available from cloned expressed genes and by using site-directed mutagenesis, some of these kinetic issues and amino acids involved may be approached in the near future.

Biochemically oriented pharmacologists have also attempted to measure the activity of GABA and related drugs at their receptors by studying the fluxes of radiolabeled chloride into or out of various types of preparations. These range from intact primary neurons and glial cells in culture (*90,91*), through neurosynaptosomal preparations in which large vesicles are prepared (*92,93*), to classical synaptosomal preparations (*94*). All of these preparations suffer from the same problem that beset the investigators who attempted similar studies of Na^+ flux from vesicles prepared from *Torpedo* membranes (*95*). That problem is simply posed in the following way: If the electrophysiological experiments clearly indicate that the chloride channel opens in response to GABA in milliseconds and demonstrable desensitization occurs on the same time scale (*85*), what relevance does measurements (with the first point on the scale of seconds-to-minutes) have to the kinetics of GABA-activated chloride conductance. It might be posed that a sort of "steady state" estimate of the final stages of the channel is reached under these circumstances; however, extensive pharmacological characterization of these systems and comparision to the steady state binding of ligands may not measure the same phenomena as the patch-clamp studies described above. Therefore, we leave the reader to examine the papers cited.

Purification of the Complex by Affinity Methods

The studies above clearly indicate that, in the membrane, the GABA–benzodiazepine receptor complex is associated with the three sets of interrelated binding sites for drugs. This complex has been solubilized sucessfully with the use of nondenaturing detergents and in different preparations has maintained many, if not all, of these regulatory sites. The first sucessful report of the solubilization of the complex involved the use of a nonionic detergent (*96*), and several of the early reports used similar detergents (*97*). More recent studies have used deoxycholate, or CHAPS (*98*) (a zwitterionic detergent), with great success in preserving regulatory interactions described above (*99–101*). Affinity chromatography using various derivatives of the benzodiazepines (most notably a flurazepam derivative) have resulted in significant purifications of the receptor proteins (*99–101*). Although no particularily novel insights into the nature of the interactions of the different binding sites have been yet derived from the study of isolated receptor, the presence of all the activities in a highly purified preparation indicates that most (probably all) of the major binding-site containing subunits of the complex have been isolated.

The general agreement is that the molecular size of the solubilized receptor is approximately 230 kdaltons (*96,100*), and the subunit structure indicates that the two major proteins identified by photolabeling are present in these preparations (*100,101, see* below). The first protein with a molecular size of 50–53 kdaltons is called the α subunit, and the slightly larger protein of 55–57 kdaltons is called the β subunit. Since both proteins appear to be isolated from bovine tissues in equal quantities, the deduced structure of the

complex then could consist of α,β dimers in duplicate. However, additional complexity of structure may be indicated by radiation inactivation experiments that indicate that the TBPS component of the receptor complex has a molecular size of 82 kdaltons, significantly different from both GABA and benzodiazepines (50 kdaltons) (*102*); a total molecular size of the complex greater than 400 kdaltons is revealed by these studies. This conclusion is based on protein target size in the membrane as opposed to hydrodynamic study of a solubilized protein. Therefore, although for this article a tetrameric complex has been assumed, it should be stated that no overwhelming evidence for this subunit stoichiometry yet exists, and other complex structures may prove to be more correct.

Monoclonal Antibodies to the Complex

Structural and Functional Studies

Antibodies have been prepared to purified GABA–benzodiazepine complexes from bovine cerebral cortex (*103–105*) and rat cortex (*106,107*). These antibodies have the properties of immunoprecipitating both GABA and benzodiazepine (photolabeled) receptor proteins from purified preparations (*103,105*) or interact with binding sites in the membrane (*108*). All of the polyclonal antibody preparations interact with the α and β subunits on Western blots of purified receptor proteins separated by SDS electrophoresis. In addition, when monoclonal antibodies were prepared (*103,104,106,107*), some of the monoclonal antibodies recognized either, and some both, the 50–53 and 55–57 kdalton proteins. From the limited information available to date, the conclusion is that both major common and unique epitopes exist on each protein. Studies of the regional distribution of these proteins on Western blots of regionally prepared samples form rat, bovine, and human brain indicate a uniform distribution of these proteins in all regions (*104*). In addition, in both rat and human cerebellum, which were postulated only to contain type 1 benzodiazepine receptors, both the α and β subunits were detected with a monoclonal antibody prepared against purified rat brain receptors (*108*); these studies would indicate that differential photoaffinity labeling with benzodiazepines may have a different basis than receptor subtypes. Regional variations in glycosylation may contribute to these differences (*109*). When antibodies are prepared against synthetic peptides containing portions of the putative receptor active sites and common regions of the α and β subunits, more interesting distinctions about structural similarities and differences of the subunits will be possible. Some changes in the binding of ligands to their binding sites have been found after treatment of the membranes with monoclonals directed against rat α and/or β subunits (*107*). These antibodies are under investigation to determine if there are intrinsic pharmacological activities of these monoclonals.

The pattern of immunoreactivity determined histochemically in rat brain with one of the monoclonals prepared against bovine receptor (*110*) shows correspondance to the autoradiographical distribution of benzodiazepine binding sites. Electron microscopic examination at a level of resolution superior to previous autoradiographic studies has led to the determination of receptor localization at a single-cell level (*110*), and extensive regional studies of the localization of the GABA receptor complex have been undertaken (*111*).

The use of monoclonal antibodies against the GABA receptor complex will also allow the examination of human brain receptors in normal states and in diseases in which previous investigation has indicated possible alterations of GABA receptors, such as Huntington's and Parkinson's diseases (*112*). Since the histochemical resolution with the monoclonals is superior to receptor autoradiography, more detailed analyses of abnormal distribution should emerge. These may suggest finer lesion studies

to be performed in experimental animals or indicate particular populations of neurons susceptable to the disease processes in humans.

Cloning of the GABA-Benzodiazepine Receptor Subunits and Anion Channels from Erythrocytes

The major subunits of the GABA–benzodiazepine receptor complex are being cloned (*113,-114*). In addition, a potentially related protein, the murine anion-exchange protein from erythrocyte membranes (band 3), has previously been cloned and sequenced (*115*). Thus, the GABA receptor subunits and an unrelated anion channel are in a position to be compared in the same way that the Na· channel and nicotinic cholinergic receptor have been studied (*5,6*). Band 3 is a major integral glycoprotein of erythrocytes catalyzing the one-to-one transport of chloride and bicarbonate. The molecular size of this protein is over 900 amino acids (molecular mass ~ 100 kdaltons) (*115*). It has been divided into three major regions, an N-terminal cytoplasmic region of 420 amino acids, a central amphipathic region of 450 amino acids containing the putative membrane-spanning region, and a carboxy terminal region that is quite hydrophilic. The sequence, PAPAKPA (similar to the photolabeled sequence above), is found in one of the presumptive extramembranous regions of this protein, on the intracellular (cytoplasmic) side. The ampipathic region of this protein contains enough residues to form the anion channel, and the sites to which stilbene disulfonate binds is in this region; stilbene disulfonates have also been shown to interact with benzodiazepine receptors (*116*).

Using *Xenopus* oocytes (*113*), the presence of mRNA coding for a number of rat brain receptor proteins, including GABA and glycine receptors, was demonstrated in fetal rat brain. This preparation presumably has genes coding for all of the possible subunits present in the normal receptor complex. No attempt at mRNA fractionation was made in this study and these mRNAs have been used as a source for the isolation of genes coding for GABA receptors. Using the monoclonal antibodies against the various receptor subunits (*114*), bacterial clones, into which similar mRNAs have been permanently incorporated, have been selected by their ability to make a bacterial protein that is immunochemically identified; in addition, partial sequence data has allowed confimation of the inserts. Sequencing and expression of these inserts in a mammalian expression system is underway (*113, 114*).

Related Proteins and Their Identification

In addition to the central benzodiazepine binding site linked to the GABA complex, there are specific, high-affinity diazepam binding sites in peripheral tissues (*117*), including the adrenal, kidney, lung, liver, testes, skin, and platelets (*118*). The benzodiazepine binding sites in peripheral tissues have pharmacological characteristics that are distinguishable from the GABA-linked benzodiazepine receptors (*119, 120*). These binding sites have high affinity for Ro5-4864, and low affinity for clonazepam, a benzodiazepine quite active at GABA-linked sites. As mentioned earlier, Ro5-4864 has little activity at central receptor sites and little pharmacological activity, except at high concentrations. The peripheral binding sites are not modulated by GABA or convulsants, indicating they are not linked to chloride channels (*121*).

This peripheral binding site has also been demonstrated in brain tissues at about one quarter the concentration of the central site; the anatomical localization of the site is quite different from the central (neuronal) site, and it has been suggested that the peripheral binding site is associated with glial elements (*120*). High levels of this site have been found in human

astrocytomas obtained at tumor removal (Tallman, unpublished), and some investigators have suggested that the site might mediate tumor growth in brain tissue (*122*).

The subcellular localization of the peripheral benzodiazepine binding site was initially assigned to the nuclear fraction (unlike the synaptosomally localized central site) [*see* ref. (*114*) for discussion]. However, autoradiographical studies in neonatal rats using tritiated Ro5-4864, suggested a correlation between high levels of these sites and high rates of oxidative phosphorylation (*118*). Thus, tissues such as kidney convoluted tubules, adrenal cortex, and olfactory epithelium contain high densities of both the peripheral sites and mitochondria; related subcellular fractionation studies indicated that, although significantly present in "nuclear fractions," the peripheral sites are more closely related to the recovery of cytochrome oxidase, as opposed to DNA. Repeated treatment of the nuclear fraction by homogenization and centrifugation transferred the sites to the "mitochondrial fraction"; thus, the binding sites appear to be associated with the mitochondria. It remains unclear whether these sites are also found outside the mitochondria.

Futher localization of the binding sites to the outer mitochondrial membrane has been indicated by detergent studies of mitochondria. The peripheral binding sites have also been estimated to compose approximately 5–15% of these proteins. Although the identity of this protein is not known at present, it would seem that the peripheral binding site is localized to an already known component of the outer mitochondrial membrane or mitochondrial matrix protein. Such a localization of the binding site to the mitochondria may explain the reported metabolic effects of the peripheral benzodiazepines (*118*).

Limited structure–activity studies have been performed for the peripheral benzodiazepines. Substitution at the no. 4 position of the phenyl ring enhances specificity for the peripheral binding site, and an *N*-methyl is necessary for optimal activity (*120*). The isoquinoline carboxamide derivative PK 11195 binds to peripheral tissues with high affinity and pharmacological binding characteristics similar to Ro5-4864 (*124*); however, Ro5-4864 binding is temperature sensitive and enthalpy driven, whereas PK 11195 binding is temperature insensitive (entropy driven) (*125*). Unsubstantiated claims about the pharmacological properties of these compounds have been made based on the thermodynamic properties of ligand binding.

A nitrophenyl derivative of PK 11195 has been developed to photolabel the peripheral benzodiazepine binding site (*126*). PK 14105 exhibits high affinity for the peripheral binding site and apparently labels the same number of sites as the other derivatives. Following photoactivation, the PK 14105 covalently labels a 18 kdalton protein and blocks the binding of other peripheral ligands. Simple one-to-one labeling and inactivation are observed. The labeling of this protein roughly corresponds with the mol wt of 23,000 obtained by radiation inactivation of peripheral binding sites (*127*). Development of an irreversible derivative of Ro5-4864 has been recently described. AHN 086 is an alkylating ligand that is a specific irreversible inhibitor of binding to peripheral benzodiazpine sites. AHN 086 has a high affinity for peripheral benzodiazepine receptors and possesses pharmacological characteristics similar to Ro5-4864; it may prove useful in further characterization of these sites (*128*).

Peripheral benzodiazepine binding sites have been solubilized in an intact form with the detergent digitonin (*126,129,130*). The digitonin solubilized peripheral binding sites migrate on gel filtration columns with an apparent mol wt of 200,000 (*126*) in contrast to the mol wt determinations of 20,000 by other methods. The peripheral binding site was found to have an isoelectric point of 4.5 (*126*), more acidic than that observed for the α subunit of the benzodiazepine receptor, which is close to 6.0 (*99,108*).

The functions of these binding sites may be associated with calcium channels. Thus, per-

ipheral binding sites have been shown to inhibit cell proliferation in mouse thyoma cell lines, whereas calcium stimulates them (*123*); they may inhibit calcium/calmodulin-stimulated membrane phosphorylation (*31*); they decrease the duration of intracellular action potentials and contractility of guinea pig papillary muscle (*132*); they inhibit endorphin release from a pituitary-derived tumor line by blockage of voltage-dependent calcium channels (*133*). The concentrations for all of these effects are much higher than might be expected from the binding characteristics of the compounds, and many could be occurring by alterations in oxidative phosphorylation. In support of calcium linkage, the calcium channel antagonists nifedipine and nitrendipine displace Ro5-4864 binding at μ*M* concentrations, but they have no effect on central binding in the same concentration range (*134*). Conversely, diazepam in the μ*M* range inhibits nitrendipine binding in guinea pig ileum (*135*). The high concentrations required indicate that the calcium antagonists may be inhibiting at a site separate from the high-affinity binding sites for these compounds. This conclusion is supported by the finding that the peripheral benzodiazepine binding site is found in very low density in nonmammalian vertebrates, whereas the dihyropyridine sites are already present in high amounts (*136,137*).

Peripheral benzodiazepine binding sites and central binding sites were not found originally in nonvertebrates. Several exceptions have now been reported. These include central type binding sites in housefly muscle preparations that are apparently linked to GABA (*138*) and mixed central/peripheral benzodiazepine binding sites (*139*). In the locust supraesophageal ganglia, flunitrazepam binding in the locust was discovered to be calcium dependent with pharamacological characteristics of both the central and peripheral benzodiazepine binding sites (*139*). This binding was enhanced by GABA, a feature of the central site, whereas Ro5-4864 was more potent than clonazepam in displacing ligand binding. Future studies carried out in the presence of calcium may reveal similar sites in other invertebrates.

Photoaffinity labeling with radiolabeled flunitrazepam in locust resulted in the labeling of two major proteins of 45 and 59 kdalton, a pattern similar to that found in the central site. Photolabeling of whole brain taken from boney fishes has also resulted in the detection of two major bands of approximately 47 and 55 kdalton. For further comparison, the influence of calcium on the binding of benzodiazepines to fish brain would be interesting. Throughout the higher vertebrates, including frog, bird, rodent, bovine, and human, the major band labeled has a molecular size of 50–53 kdalton. The most notable additional band is a protein of 55–58 kdalton and usually there is a detectable band at 47 kdalton in these species (*104–142*) (*see* Table 1). It is not clear yet whether this band is identical to the protein of 55–58 kdalton, and usually there is a detectable band at 47 kdalton in these species (*140–142*). It is not clear yet whether this band is identical to the protein detected in invertebrates, is a breakdown product of the other higher molecular mass proteins, or is an entirely different protein.

Photoaffinity labeling of neonatal rat brain has revealed that the major band in adults does not appear until after d 20 of gestation (*62*). In contrast, photolabeled proteins of 55, 59, and 63 kdalton predominate at earlier time periods. The levels of the 59 kdalton protein decline as the animals mature. The earliest appearance of the high molecular weight species of GABA–benzodiazepine receptors may be a case of "ontogeny recapitulating phylogeny" or may indicate forms of the receptor present during fetal but not adult life (reminicent of fetal hemoglobins). Immunoblot experiments of the invertebrate sites and developmentally early forms of the receptor complex remain to be studied. In addition, the composition of the genes coding for the different sized receptors and their relationships to the phylogenetic placement of the species should also be examined with appropriate gene probes for each subunit.

Glycine/Strychnine/Chloride Channel

Introduction

Glycine is now thought to be the major inhibitory amino acid transmitter in the spinal cord and brainstem of higher vertebrates (*143*). Evidence for this role has been derived from the use of traditional neurophysiological and neurochemical approaches (*144*). Endogenous glycine levels are highest in the gray matter of the spinal cord and medulla, and levels decrease acutely as one moves rostrally through the neuroaxis; in the glycine-rich regions, a sodium-dependent, high-affinity uptake system for glycine has been found (*145*). The glycine accumulated in these regions shows fairly selective release by either electrical or potassium-induced depolarization (*146,147*). Neurophysiologically, it has been shown that glycine mimics the action of the endogenous inhibitory neurotransmitter in the spinal gray matter and areas of the medulla; in these areas, glycine increases

TABLE 1

[^{3}H]Flunitrazepam Photolabeling of Benzodiazepine Binding Proteins in Different Species

	Benzodiazepine binding protein, mol wt				
Species	45,000–47,000	50,000-53,000	55,000–58,000	60,000+	Ref.
Locust	+++[a]	-	+++	–	*136*
Boney fish	+++	+	+++	–	*137*
Frog	+	+++	–	–	*137*
Avian	+	+++	–	–	*137*
Neonatal rat hippocampus	–	+	++	+	*59*
Rat	+	+++	+	–	*50,51*
Mouse	–	+++	–	–	*137*
Guinea pig	+	+++	++	–	*138*
Bovine	+	+++	++	–	*137,139*
Human	+	+++	+	–	*140*

[a]+, Relative intensity of photolabeled bands within species.

chloride conductance (*148,149*). The existence of distinct tracts for glycine in the brain has been difficult to establish because glycine is so widely used for common metabolic functions.

Molecular Topology of the Glycine–Chloride Complex

Since glycine is present in quite high concentrations in the spinal cord and because the affinity for its receptor is in the μ*M* range, the biochemical demonstration of a receptor site for this transmitter depended upon the discovery that the alkaloid strychnine appeared to cause convulsions by blocking glycine's inhibitory action in the spinal cord (while not inhibiting presynaptic uptake sites). Using tritiated strychnine, it has been possible to localize autoradiographically glycine receptors in the brain and to undertake a neurochemical analysis of ligand–receptor interactions (*150*).

Strychnine binds to spinal cord membrane fractions in a highly selective and saturable manner, with a K_d of about 30 n*M* and a Hill coefficient of 1 (*151*). Glycine displaces the binding of strychine with an affinity of approximately 10 μ*M* and with a Hill coefficient of about 2. Thus, it appears that there are mutually interacting but separate sites on the complex for the agonist glycine and the antagonist strychnine; this is perhaps analogous to or reminiscent of the interactions of GABA and β-carbolines or TBPS. The Hill coefficient of 2 also indicates that, like GABA, more than one molecule may bind to the receptor complex at one time or that several affinity states for the agonist may exist within the same complex (*152*). It is not clear at present whether these are distinct domains on the same protein or are on separate isolated putative subunits of the receptor (*see* below).

Two other inhibitory substances interact with the glycine receptor; they are taurine and β-alanine (*153*). In contrast, GABA has little activity at these sites. Like glycine, β-alanine and taurine are relatively simple amino acids with some structural similarity to glycine. Since these amino acids are present in many parts of the brain where strychnine binding is not present, they may also possess their own separate receptors; for example, it has been suggested that taurine may be an inhibitory transmitter in the retina (*153*). The status of these putative transmitters is unclear at present. The relative paucity of drugs with activity at the glycine receptor has limited the molecular pharmacology of this area and not allowed the complete characterization of the topology of this receptor. Recent analogs of THIP with glycine antagonist activity may partly fill this void (*24*).

As in the case of the benzodiazepine receptor, chloride can interact with the binding site of the glycine complex to inhibit strychnine binding in a noncompetitive fashion (*152*). This has led some investigators to postulate that the strychnine binding site is intimately connected with the chloride conductance mechanism. Because the stoichiometry and nature of the inhibition is not fully understood, it is impossible to determine if strychnine binds to a domain that actually comprises the "chloride channel" or a domain that can interact with the channel's ionic components.

Details of Structure from Patch-Clamp Studies

Studies of glycine receptor function have also been carried out using patch-clamp and fluctuation analysis in spinal cord cultured neurons (*83,87,89,154*). The consensus of these studies was that several of the conductance states of the chloride channel were shared between GABA and glycine receptors. In addition, in some patches only glycine responses have been observed, whereas in others a GABA response was seen. Glycine causes desensitization in the μ*M* range, as described above for GABA, and cross-desensitization between GABA and glycine has been observed (*87*). This indicates that in some

cells the two transmitters may share chloride channels (*87,89,154*). However, the multistate behavior can still be observed in the presence of specific blockers of the individual receptors, indicating that the responses may not be from the identical receptor subunits; in addition, glycine responses are not seen at all in adrenal chromaffin cells (*88*). One possibility is that the GABA and glycine receptors control a single type of channel and another is that they can form heteroligomers between one another (*see* below for a full description of this possibility after the subunit structure of the glycine receptor is discussed).

Molecular Characterization and Purification of Glycine Receptors

Attempts have been made to purify the glycine receptor and prepare monoclonal antibodies to the purified preparations. These studies have paralleled the work on the GABA receptor and show some similarities between these different chloride gating receptors. Using the nonionic detergent Triton X-100, solubilization of greater than 50% of the glycine receptors (strychnine binding sites) was obtained from rat spinal cord (*15*). Using an affinity column containing 3-aminostrychnine linked through a 10-C spacer, extensive purification of the glycine receptor from rat and pig spinal cord has been obtained (*156*). PAGE electrophoresis in the presence of SDS under reducing conditions indicated that the purified preparations contained three major proteins of 48 (α), 58 (β), and 93 kdalton molecular mass. Current investigations indicate that the 93 kdalton protein is loosely associated with the complex and is a peripheral membrane protein that can be removed from the membrane with gentle treatment; therefore, it is unlikely to be the chloride channel (*157*). Gel filtration and sucrose density gradient centrifugation estimate the mol wt of the intact complex to be approximately 250,000. Perhaps the stoichiometry of this complex can best be represented by a tetrameric structure containing two α and two β subunits (*158*). This would be quite similar to the proposed structure of the GABA–benzodiazepine complex.

Using radiolabeled strychnine, it has been possible to photolabel the glycine receptor complex. Following separation of the subunits on SDS gel electrophoresis, the predominant labeling was found in the 48 kdalton subunit (*159*). This photolabeling could be blocked both by strychnine and glycine. Tryptic digestion of the photolabeled membranes apparently did not release small, labeled peptides into the supernatant, as in the case of the benzodiazepine receptor (and only reduced the molecular size by 20%). Perhaps more extensive degradation is required for release, or it is possible that the photolabled strychnine site is associated with a hydrophobic portion of the molecule and is inaccessable to proteolytic degration. Endoglycosidase treatment did not alter the apparent size of the receptor subunit labeled with strychnine; however, the use of lectin affinity columns in later stages of purification indicated that the glycine receptor complex was glycosylated (*158*).

Antibodies to the Glycine Receptor

Nine different monoclonal lines were established that secreted antibodies directed against the purified glycine receptor complex from rat spinal cord. Only six of these lines recognized the denatured proteins on Western blots. Two of the lines recognized more than one of the three bands. One monoclonal antibody recognized the 48 and 58 kdalton bands and another the 93 and 48 kdalton bands (*160*). Limited peptide mapping of the three proteins revealed each protein had common molecular-sized peptides recognized by the monoclonals. These data suggest that the three proteins may possess significant homology in their primary structure.

These monoclonal antibodies have also been used to study the topology and function of the glycine receptor complex. In mature murine neuronal cultures, glycine receptor-like immunoreactivity obtained with one of the antibodies revealed a nonuniform distribution on the cell surface, perhaps indicating a neurotransmitter receptor action. This antibody showed some pharmacological activity through its ability to activate the glycine complex as measured electrophysiologically (*161*).

These monoclonals could also serve for screening cDNA libraries and help to isolate genes coding for the proteins that comprise the glycine/strychnine/chloride ionophore complex. This research would be important for preparing large amounts of the glycine receptor complex for biochemical studies and also for ascribing function to each of the subunits. Since fluctuation and patch-clamp analysis of GABA and glycine responses indicate that these receptors share common conductance states, it is likely that genetic analysis would detect significant homologies between the subunits of each complex. Such similarities can already be surmised from the common drugs that can interact with each site. For example, avermectin B1a, an antihelmintic drug, interferes with both GABA receptor (barbiturate/TBPS site) and strychnine binding (*162*). It was also originally postulated that the benzodiazepines interacted with the glycine receptor to produce their actions (*152*); although not borne out by the extensive investigation into the molecular actions of benzodiazepines that has followed, these experiments may indicate some affinity of the glycine receptor for the benzodiazepine molecule. Similar weak cross-talk between barbiturates, picrotoxin, and strychnine itself lends support to the relation of the component subunits of both complexes.

It is possible that enough homology exists to form heterotetramers with complex structure. Thus, in addition to receptor complexes that respond to either GABA or glycine (and their antagonists, and the like), complexes containing one of each of the four subunits of the collective GABA and glycine receptor may exist. In cells expressing both GABA subunits (α and β) and glycine subunits (α' and β'), the possible structures might be $(\alpha,\beta)_2$ $(\alpha',\beta')_2$ or $\alpha,\alpha',\beta,\beta'$. Since the subunits may be sufficient to form the channel itself, these would be true isoreceptors and capable of responding to both GABA and glycine at the same channel. Molecular biological studies using expression systems and patch-clamp recording after introduction of mixtures of genes coding for each subunit α and β of either GABA or glycine receptors should help examine this possibility. A picture of the possible arrays of subunit structure in cells expressing both sets of genes is shown the Fig. 6.

Conclusions and Future Directions

This review has attempted to integrate a large number of molecular studies into a coherent view of the GABA–benzodiazepine receptor complex. With the cloning of the receptor protein and attendant determination of the primary amino acid sequence of the α and β subunit of the GABA and glycine receptors, we are in a position to embark on a series of experiments far beyond what is possible in traditional molecular pharmacology.

There are several possibilities. Site-specific mutagenesis can be used to determine the effects of introducing point mutations into the subunits at critical sites for activity. Chimeric molecules, incorporating amino acid inserts or deletions of regions of the receptor ,can also be easily formed. Since these proteins can be incorporated into cells, it will be possible to study their pharmacological properties from a functional and ligand-binding perspective. Interesting receptor molecules can then be used to form transgenic animals with "genetic diseases" of the GABA complex.

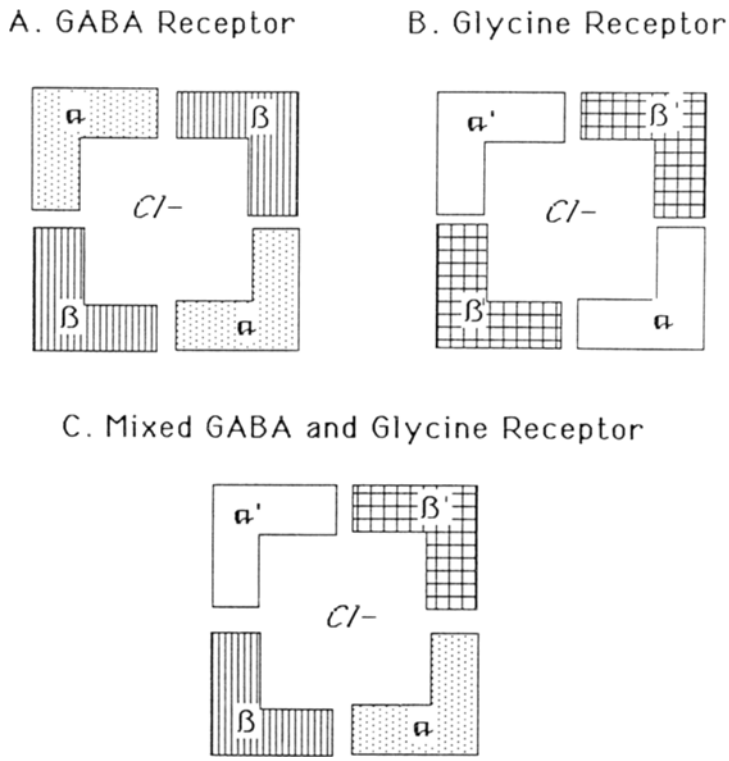

Fig. 6. GABA– and glycine–receptor /chloride ionophores may be tetramers composed of two different dimers. Cells expressing both GABA (α and β) and glycine (α' and β') subunits could have receptors composed of several different structures. Sufficient homology to accomplish this is suggested by pharmacological and electrophysiological studies described in the text.

Some of these studies may be directed by the clinical studies that are now possible. With genetic probes for the GABA–complex, it is possible to study the chromasomal localization of the GABA– and glycine–complex subunits in humans and determine whether the receptors or subunits are linked. Major polymorphisms of the GABA receptor subunits in humans can be found by molecular genetics techniques. Linkage of these multiple molecular forms of the receptor with susceptability to seizures (for example) can be studied. Rare alleles coding for inactive or deficient receptors can be found and linked to disease processes. These rare alleles can be created in vitro and the effects of genetic mutation on protein activity extensively studied, as described above.

Families of related proteins and forms of the receptor expressed during development can be identified. Possible phylogenetic origins of the GABA– and glycine–complexes can be uncovered and hitherto covert relations can be uncovered.

The ability to produce individual subunit proteins in large quanity also allows a number of experiments that have not been possible until

present. These include the study of the stoichiometry of the complex, functions of the individual subunits (which receptor is located where), and kinetic studies with fluroescence techniques requiring greater than normally available numbers of protein molecules.

It should also be possible to prepare a second improved generation of antibodies with known peptide and subunit specificities. These antibodies can be used to further resolve the immunocytochemical localization of the receptor complexes. Additional histochemical (*in situ* hybridization) and wet chemical evaluation of mRNA levels coding for receptor and measuring turnover of mRNA after drug manipulations will allow study of the effects of chronic drug administration at a regional and cell specific level of resolution.

Finally, in pharmaceutical development, modeling of the contours of the individual subunits could lead to synthesis of drugs with subunit specificity and activity against defined portions of the receptor molecule. In the most optimistic world, several of the approaches could be combined and drugs with specificity against proteins produced by site-specific mutagenesis could be synthesized. If these molecules were in turn modeled upon abnormal proteins in people, the ideal pharmcological agent with specificity largely toward abnormal receptors could be created. Such molecules will be the products of the 21th Century.

Many important discoveries remain in this and other receptor systems!

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