Molecular Structure and Function of the Glycine Receptor Chloride Channel

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modulatory effects on this receptor. Since GlyRs are involved in motor reflex circuits of the spinal cord and provide inhibitory synapses onto pain sensory neurons, these agents may provide lead compounds for the development of muscle relaxant and peripheral analgesic drugs.

I. INTRODUCTION

A. Scope of This Review

Ligand-gated ion channels permit cells to respond rapidly to changes in their external environment. They are particularly well known for mediating fast neurotransmission in the nervous system. The glycine receptor (GlyR) is a membrane-embedded protein that contains an integral Cl⁻–selective pore. When glycine binds to its site on the external receptor surface, the pore opens allowing Cl⁻ to passively diffuse across the membrane. The GlyR is a member of the pentameric ligand-gated ion channel (LGIC) family, of which the nicotinic acetylcholine receptor channel (nAChR) is the prototypical member. Other members of this family include the cation-permeable serotonin type 3 receptor (5-HT₃R), anion-permeable GABA type A and C receptors (GABAₐ,R and GABAₐ,R), recently identified cation-permeable zinc and GABA receptors (34, 86), as well as invertebrate anion-permeable glutamate and histidine receptors (130). Note that glycine also directly activates a cation-selective ion channel of the excitatory glutamate receptor family (62). The structural and functional properties of this receptor class have recently been reviewed (94) and are not considered here.

Glycine was first proposed as an inhibitory neurotransmitter on the basis of a detailed analysis of its distribution in the spinal cord (16). Subsequent electrophysiological studies demonstrated a strychnine-sensitive hyperpolarizing action of glycine on spinal neurons (80, 395). This hyperpolarization was soon discovered to be mediated by an increase in Cl⁻ conductance (81, 82, 396). The receptors responsible for these actions were subsequently purified by strychnine affinity chromatography (291, 292), and the first GlyR subunit was cloned in 1987 (138).

Current research into the GlyR can be divided into two major strands. The first involves the investigation of the molecular mechanisms of GlyR trafficking and clustering at synapses. This area is currently the subject of intense investigation, and recent progress has been covered in several authoritative reviews (189, 190, 219). The second research strand is concerned with understanding the molecular structure and function of the GlyR. Research has intensified in this area over the past few years, and the purpose of this review is to present a coherent view of recent findings. Much of our understanding of GlyR structure-function has been gained by comparison with the structurally homologous nAChR. Hence, this review makes frequent references to research on the nAChR, particularly in areas where knowledge of the GlyR is deficient.

B. Glycine as an Inhibitory (and Excitatory) Neurotransmitter

When the GlyR is activated, the resulting Cl⁻ flux moves the membrane potential rapidly toward the Cl⁻ equilibrium potential. Depending on the value of the equilibrium potential relative to the cell resting potential, the Cl⁻ flux may cause either a depolarization or a hyperpolarization. The GlyR is generally known as an inhibitory receptor because the Cl⁻ equilibrium potential is usually close to or more negative than the cell resting potential. Subthreshold depolarizations can inhibit neuronal firing if they are accompanied by an increase in membrane conductance that shorts out excitatory responses, a phenomenon termed “shunting inhibition.” However, in embryonic neurons, the intracellular Cl⁻ concentration is raised substantially, with the effect that GlyR activation causes a strong, suprathreshold depolarization. These large glycine-induced depolarizations gate a calcium influx that is necessary for the development of numerous specializations, including glycinergic synapses (190). The switch to the mature neuron phenotype is mediated by the expression of a K⁺–Cl⁻ cotransporter, KCC2, which lowers the internal Cl⁻ concentration, thereby shifting the Cl⁻ equilibrium potential to more negative values and converting the actions of the GlyR from excitatory to inhibitory (355).

II. DIVERSITY, DISTRIBUTION, AND FUNCTION OF GLYCINE RECEPTOR SUBUNITS

A. Molecular Diversity

Betz and colleagues (291, 292) originally purified the rat spinal cord GlyR by affinity chromatography on aminostrychnine-agarose columns. Oligonucleotides designed from peptide sequences of purified receptors were then used to probe a rat spinal cord cDNA library, resulting in isolation of cDNA clones corresponding to the 48 kDa (α₁) and 58 kDa (β) subunits (137, 138). Subsequently, cDNAs of the rat α₂- and α₃-subunits were cloned by homology screening (9, 199, 201). The α₄-subunit, which does not appear to exist in the rat or human, was first identified in the mouse (254) and has subsequently been found in the chick (157) and zebrafish (91). While the
α-subunit genes are highly homologous, with primary structures displaying 80–90% amino acid sequence identity, the β-subunit has a sequence similarity of ~47% with the α1-subunit (137).

The rat α1-subunit has a splice variant, termed α1ins, which contains an eight-amino acid insert in the large intracellular loop (244) that contains a possible phosphorylation site (see sect. VI.A). Alternative splicing of the rat α2-subunit generates two splice variants, α2A and α2B (199, 201). The α2B-variant differs from α2A by the V58I and T59A amino acid substitutions. Another version of the α2-subunit, termed α2*, incorporates a single amino acid substitution (G167E) that confers strychnine insensitivity (91). Although a human contains a 15-amino acid insert in the ligand-binding domain (199, 201). The α3-subunit genes are highly homologous, with primary structures displaying 80% amino acid identity (91). Although a human β-subunit gene polymorphism has been described (264), it does not appear to result in a coding mutation.

All GlyR genes cloned to date share a similar exon-intron organization with the coding region spread over nine exons (254, 264, 346). This common organization suggests a phylogenetic gene duplication (345).

B. Distribution and Function in the Rat Nervous System

1. Distribution of functional GlyRs

A) DISTRIBUTION OF STRYCHNINE AND GLYCINE BINDING SITES. Autoradiographic localization of [3H]strychnine binding sites was first studied by Zarbin et al. (431) in the rat. Strychnine binding sites were shown to exist at high levels in the spinal cord and medulla and at lower levels in the pons, thalamus, and hypothalamus, while being virtually absent in higher brain regions. In the spinal cord, their distribution is relatively diffuse throughout the gray matter. In contrast, GlyRs in the brain stem are highly localized to discrete nuclei, notably the trigeminal nuclei, the cuneate nucleus, the gracile nucleus, the hypoglossal motor nucleus, the reticular nuclei, and cochlear nuclei (301). The retina, which also has a high concentration of strychnine sites (297), is considered as a separate case, below. Together, these areas comprise a subset of the distribution as determined by glycine autoradiography (270) or glycine immunoreactivity (310), a mismatch that is understandable given that glycine is also associated with glutamatergic synapses (270). An advantage of strychnine autoradiography is that it reveals the presence of surface-expressed receptors, but a disadvantage is that its limited resolution does not permit the ultrastructural localization of strychnine binding sites. Thus strychnine autoradiography does not necessarily define the distribution of glycineric synapses.

B) DISTRIBUTION OF GLYR IMMUNOREACTIVITY. Many studies have examined the immunocytochemical localization of GlyRs at the light and electron microscopic levels using generic GlyR α-subunit monoclonal antibodies. At the light microscopic level, there is a strong correlation between the distribution patterns revealed by GlyR immunoreactivity and strychnine autoradiography (17, 370). However, some significant differences have been observed. First, immunolabeling reveals the existence of GlyRs in the cerebellum (17, 361) and olfactory bulb (383), whereas none was seen using strychnine binding (431). Second, the substantia gelatinosa in the spinal cord is strongly labeled by strychnine (431), but not by GlyR antibodies (23). The reasons for the differential labeling are yet to be clarified.

Electron microscopic immunoreactivity reveals that GlyRs at central synapses are concentrated into regions closely apposed to presynaptic terminals (13, 23, 370, 371, 383), strongly suggesting a functional role. It is of interest to note that this approach has demonstrated the colocalization of GlyRs and GABAARs at individual postsynaptic densities in the spinal cord (43, 127, 369) and cerebellum (100).

C) DISTRIBUTION OF FUNCTIONAL GLYCINERGIC SYNAPSES. Most central nervous system neurons are inhibited by glycine (279). Of course, the mere presence of functional GlyRs, especially on dissociated or cultured neurons, does not imply a physiological role. However, it has recently been proposed that the activation of nonsynaptic GlyRs in embryonic cortical neurons may be important for development, and that taurine released from local glial cells may be the endogenous ligand (114). Similarly, nonsynaptic GlyRs on hippocampal CA3 neurons are proposed to be held in a tonically active state by locally released taurine or β-alanine (273). The idea that glycineric ligands may act on nonsynaptic GlyRs to mediate processes of physiological importance certainly warrants further attention. Traditionally, however, a functional role for GlyRs in neurons has required the demonstration of strychnine-sensitive synaptic currents.

There is abundant evidence for the existence of functional glycineric synapses in the retina (see below) in spinal cord motor reflex pathways (219) and in spinal cord pain sensory pathways (4). Glycinergic neurotransmission has also been demonstrated in various brain stem nuclei. For example, it has been well characterized in several brain stem nuclei of the central auditory pathways. In the medullary cochlear nucleus, which receive inputs directly from the auditory nerve, glycineric synapses occur onto stellate cells (108) and bushy cells (230). In the trapezoid body, a subsequent major relay station in the auditory pathway, GlyRs are located presynaptically at calyceal synapses onto principal cells (373). The prom-
inent output from this nuclei extends to the superior olivary complex of the pons, where glycineric synapses are also found (194, 351). Functional glycineric synapses also exist on neurons in the medullary trigeminal (421), abducens (325), and hypoglossal motor nuclei (248, 347, 375). In the cerebellum, glycineric synapses mediate inhibitory neurotransmission between Lugaro cells and Golgi cells in the cerebellar cortex (93), and between interneurons and principal cells in the deep cerebellar nuclei (182). This list may expand as other brain stem nuclei are characterized in detail.

It is relevant to note that glycine may not be the sole inhibitory neurotransmitter at many of these synapses. Mixed GABA-glycine synapses may mediate neurotransmission at individual synapses in the spinal cord (174), brain stem (194, 283, 325), and cerebellum (100). Interestingly, GlyR activation appears to be able to inhibit GABA<sub>R</sub>s via a phosphorylation-dependent mechanism (229). This process may be important for regulating inhibitory synaptic current magnitude at mixed GABA-glycine synapses. There is evidence that the GABAergic component of inhibitory neurotransmission at mixed synapses may be upregulated in individuals suffering from heritable disorders of glycineric neurotransmission (see sect. VII).

Finally, presynaptic GlyRs have been functionally characterized at calyceal synapses (373) and on terminals synapsing onto rat spinal sensory neurons (172). Surprisingly, in both cases GlyR activation is excitatory, leading to increased neurotransmitter release.

2. Distribution of GlyR subunits

In situ hybridization was the first approach employed to localize the distribution of individual GlyR subunits in the rat. An advantage of this approach is its subtype specificity, but a disadvantage is that transcript expression does not necessarily imply the surface expression of functional receptors. Expression of α1-subunit mRNA in adult rats was highest in the brain stem nuclei and spinal cord, but it was also found in the superior and inferior colliculi and in regions of the thalamus and hypothalamus (245, 331). It was notably absent from cortical regions. Thus, with few exceptions, its distribution is similar to that of functional GlyRs as described above. In the rat, expression is detectable at embryonic day 15 and increases to a maximum at around postnatal day 15, without substantial changes in its distribution (245). Northern blot analysis reveals that α1<sub>1</sub><sup>ins</sup> shares a similar distribution (244).

Prenatally, transcripts of the α2-subunit gene are found throughout most of the central nervous system. However, postnatally they decline sharply with little label remaining by postnatal day 20 (9, 245, 392). Detectable amounts of α2-transcripts do persist into adulthood, however, notably in the retina (see below), auditory brain stem nuclei (293), and some higher brain regions (245). The α2A-isoform is expressed more abundantly than α2B during development, although the α2B-isoform is present at higher levels in the adult (199).

The distribution and developmental changes in α3-transcripts generally resemble those of α1-transcripts, with the exception that α3-expression is much less intense at all developmental stages (245). As with the α1-subunit, its expression intensity increases postnatally to reach a maximum at around 3 wk (245). The α3L- and α3K-variants share similar distribution patterns (280). GlyR α4-subunit transcripts are expressed at very low levels (if at all) in the adult rat (293), although they are strongly expressed in the spinal cord, dorsal root ganglia, sympathetic ganglia, and the male genital ridge of the chick (157).

GlyR β-subunit transcripts are distributed widely throughout the embryonic and adult central nervous system (125, 245). Although present at low levels prenatally, expression increases dramatically after birth and persists into adulthood (245). The reason for this broad expression profile is somewhat puzzling given that these subunits do not form functional receptors in the absence of α-subunits.

3. Developmental switch from α2 to α1β

Becker et al. (27) showed using protein expression that fetal GlyRs are predominantly α2-homomers, whereas adult receptors are predominantly α1β-heteromers. Such a switch is also supported by the mRNA expression patterns described above. In the neonatal rat, the α1-, α2-, and β-subunits exist in abundance, implying a mixture of receptor isoforms, but the switch towards the adult isoform is complete by around postnatal day 20 (27, 121, 392). The sparse expression of the α3- and α4-subunits suggests they may also be included in a minority of adult GlyRs. Recent evidence suggests this switch may not be as complete as originally thought and that α2-subunit expression may remain at significant levels throughout adulthood in the retina (see below) and auditory brain stem (293). Although the mechanism responsible for triggering the developmental switch is not known, it does not seem to require the activation of the GlyRs themselves (247).

Given that α2-subunits alone are expressed in embryonic neurons, is it possible that homomeric α2-GlyRs may mediate synaptic transmission? Takahashi et al. (361) showed that the single-channel conductance and kinetic properties of recombinant homomeric α2- and α1-GlyRs were similar to those of native GlyRs in rat spinal neurons at embryonic day 20 and postnatal day 22, respectively. They also demonstrated an increased decay rate of the glycineric inhibitory postsynaptic currents (IPSCs) over the same period that was consistent with the change in
channel kinetic properties (361). Subsequent studies have supported these findings (12, 347). However, it is unlikely that homomeric α2-GlyRα2-GlyRs mediate inhibitory neurotransmission for the following reasons. First, because β-subunits are required for GlyR postsynaptic clustering (188, 259), it is not certain how the α2-homomers would undergo the prerequisite aggregation at postsynaptic densities. Second, a recent study has found that α2-homomeric GlyRs activate too slowly to effectively mediate synaptic transmission (246). Given their wide distribution throughout the nervous system during development, and the fact that Cl⁻ fluxes are excitatory in developing neurons (114, 355), it seems more likely that homomeric α2-GlyRs mediate nonsynaptic cell-to-cell communication that could be important for neuronal differentiation and synaptogenesis (190). Glycinergic synapses in immature neurons are probably comprised of α2β-heteromeric GlyRs (219). The single-channel conductance of synaptic GlyRs from embryonic neurons is consistent with such a conclusion (12, 347).

4. A special case: the retina

This is considered separately because the profile of GlyR subunit distribution is atypical and has been mapped in detail and because a specific role for glycineric synapses has been proposed. GABA and glycine both function as inhibitory neurotransmitters in the retina (143, 176, 297, 390). In situ hybridization and immunohistochemistry both show that GlyR α1-, α2-, α3-, and β-subunits have different patterns of distribution in the adult rat (136, 146, 330). The α1-subunit is distributed predominantly on bipolar cells and on some ganglion cells in the inner plexiform layer (136, 144, 145, 231). The α2-subunit is distributed on amacrine cells and on almost all ganglion cells, whereas the α3- and β-subunits are distributed widely throughout the inner plexiform layer (136). A detailed study in the mouse concluded that α1-subunits are associated with synapses in the rod pathway between all amacrine cells and off-cone bipolar cells, whereas α3-subunits are restricted to cone pathways (159). Together these results indicate a spatial distribution of GlyR subunit composition throughout the adult retina. Consistent with this picture, Enz and Bormann (105) detected mRNA for all four GlyR subunits in RNA from whole retina, but mRNA for only α1- and β-subunits in RNA isolated from individual rod bipolar cells.

Electron microscopy has confirmed that the punctate immunoreactivity seen with the light microscope is due to clusters of GlyRs at the postsynaptic densities (146, 330). Consistent with these anatomical studies, electrophysiological investigations in the rat have revealed the presence of glycineric inhibitory postsynaptic potentials (IPSPs) in identified amacrine cells (119), ganglion cells (153, 302, 368), and rod bipolar cells (77, 99, 303).

The synaptic distribution of GlyR subunits is spatially distinct from that of GABAα2-GABAα2-R subunits, although individual ganglion cells may possess both types of synapse (195, 329). Recent studies have begun to address the possibility that glycineric and GABAergic transmission may have distinct physiological roles. Although functional differences between GABAergic and glycineric IPSPs in retinal neurons have been demonstrated (119, 302), the physiological significance remains unknown. However, structural studies have provided evidence that the GABA and glycine synaptic pathways participate in different functional circuits. In particular, glycineric synapses are thought to play a specific role in the transmission of dark-adjustment signals through the off-channel of the rod pathway from amacrine cells to off-bipolar cells and hence to off-ganglion cells (146, 330, 391). This circuit contributes to the switch from day to night vision.

C. Distribution and Function in Other Tissues

1. Spermatozoon

The front of the mammalian sperm head contains a large secretory vesicle termed the acrosome. The process of fertilization is initiated when the sperm head contacts the outer coat, or zona pellucida, of the egg. A zona pellucida-specific glycoprotein, ZP3, forms the sperm receptor. Its interaction with sperm initiates a complex intracellular signaling mechanism inside the sperm that culminates in a calcium elevation that is thought to be mediated at least partly by an influx through voltage-gated calcium channels (115). This event, termed the acrosome reaction (AR), results in the release of acrosome hydrolytic enzymes by exocytosis. These enzymes induce various protein modifications to ensure that the sperm remains tightly bound to the zona pellucida while fusion takes place between the sperm and egg plasma membranes.

The activation of GlyRs and GABAα2-Rs in the sperm plasma membrane appears to be essential for the AR (257). There is considerable evidence that GlyRs exist in sperm plasma membranes. For example, immunohistochemical studies have demonstrated the existence of GlyR α- and β-subunit protein in porcine, mouse, and human sperm (49, 258, 332). An immunofluorescence study localized the α-subunits to cell membranes in the periacyrosomal regions of live mouse sperm (332). In addition, strychnine binding studies have revealed the presence of GlyRs in hamster sperm (232).

Functional evidence for GlyR involvement has also been demonstrated. For example, glycine initiated the AR in a manner that was inhibited by strychnine or a GlyR α-subunit antibody (49, 332). Furthermore, studies using fura 2-loaded human sperm showed that 50 nM strychnine was also able to inhibit the ZP3-mediated calcium influx...
(49). Finally, sperm from homozygous spasmodic and spastic mice (which possess defective GlyR α1- and β-subunits, respectively) are deficient in their ability to undergo the AR (333).

Thus the GlyR is likely to have a central role in the AR. However, two questions remain about this process. What is the concentration of Cl⁻ inside sperm? Presumably, it must be high enough to force an outward (i.e., depolarizing) Cl⁻ flux upon GlyR activation. Second, what is the glycine concentration in the oviduct where fertilization takes place? Do the GlyRs remain tonically active holding the sperm in a depolarized state preceding the AR?

Harvey et al. (157) found that the α4-subunit gene is expressed on the developing male genital ridge of the chick and proposed that GlyRs containing this subunit may contribute to the development of immature spermatogonia.

2. Endocrine pancreas

A pancreatic cell line, GK-P3, expresses functional GlyRs. When activated, these receptors cause a depolarization that increases the intracellular calcium concentration (393). A glycine receptor antibody displayed immunocytochemical methods revealed the presence of GlyRs in pancreatic islet cells (393) prompting the authors to surmise that GlyRs may also be expressed in islet cells in vivo. However, there is as yet no electrophysiological evidence for GlyRs in pancreatic islet cells.

3. Adrenomedullary chromaffin cells

High-affinity [³H]strychnine binding sites have been shown to exist in catecholamine-secreting chromaffin cells of the adrenal medulla (415, 416). The same group subsequently demonstrated that glycine can stimulate significant catecholamine secretion from chromaffin cells in both in vitro and in vivo assays (414, 417). The presence of GlyR α3-subunit mRNA (but not α1 or α2) was also demonstrated by RT-PCR from RNA extracted from rat adrenal glands. However, direct electrophysiological evidence for glycine-activated currents in chromaffin cells is conspicuously absent to date.

4. Kupffer cells and other macrophages

A variety of pharmacological evidence, summarized in Reference 184, suggests that GlyRs may at least partially mediate the anti-inflammatory effects of glycine in macrophages and leukocytes. Research on GlyR involvement in these processes has focused on Kupffer cells, which are specialized macrophages found in the liver. Glycine has been shown to reduce the magnitude of lipopolysaccharide-induced calcium transients in these cells in a strychnine-dependent manner (122, 167). Similar observations have also been made in neutrophils (398) and hepatic parenchymal cells (304). Recent evidence from Western blots, RT-PCR, and RNAse protection assays indeed suggest the presence of GlyR α1-, α2-, α4-, and β-subunits in Kupffer cells (122).

5. Neural stem progenitor cells

Strychnine-sensitive glycine-gated currents are present in postnatal, nestin-positive neural stem progenitor cells (278). Consistent with this observation, RT-PCR and immunocytochemical methods revealed the presence of α1-, α2-, and β-subunit RNA transcripts and α-subunit protein, respectively.

III. STRUCTURE AND ASSEMBLY

A. General Structural Features

The nAChR is the most intensively investigated member of the LGIC family. Consequently, most of the structural features of the GlyR have been deduced from its homology with this receptor. LGIC receptors contain five subunits arranged pseudo-symmetrically around a central ion-conducting pore. The membrane topologies of all LGIC subunits are similar. This topology includes a large NH₂-terminal extracellular domain that contains the agonist binding sites. A defining feature of LGIC subunits is the conserved cysteine loop in this domain. All GlyR subunits also harbor a second cysteine loop (309) that incorporates a principal glycine-binding domain. As discussed in detail below, the crystal structure of acetylcholine-binding protein (AChBP) provides an excellent model for understanding the structure of this domain (52).

Hydropathy analysis originally predicted an arrangement of four α-helical transmembrane domains (TM1–TM4) per subunit. Although evidence exists for the inclusion of β-sheet in the TM regions (134), the recent elucidation of the crystal structure of the Torpedo nAChR TM domains provides an overwhelming argument in favor of the original four α-helical model (267). This structure, determined by cryoelectron microscopy to a resolution of 4 Å by Miyazawa et al. (267), is a major advance in the field. Finally, TM3 and TM4 are linked by a large, poorly conserved, intracellular domain that contains phosphorylation sites and other sites for mediating interactions with cytoplasmic factors. The structure and function of each of these regions is now considered in detail.

B. Transmembrane Domains

1. Spatial organization

The principal role of the TM domains is to provide a sealed barrier to separate the ion permeation pathway
from the apolar region of the lipid bilayer. In most ion channels of known structure, this is achieved by a close packing of amphipathic α-helices at angles close, but not quite perpendicular, to the plane of the membrane (353). This arrangement also applies to LGIC receptors, with each subunit contributing an α-helical TM2 domain to the lining of a single central water-filled pore. The TM1, TM3, and TM4 domains surround TM2 and provide the interface with the lipid bilayer, thereby isolating TM2 from direct contact with the surrounding hydrophobic environment. Viewed from the synapse, TM1–TM4 are arranged consecutively in a clockwise manner, with TM1 and TM3 located closest to TM2 (267). In the nAChR, the TM domains splay outwards towards the extracellular membrane surface and extend about two helical rotations (~10 Å) beyond the hydrophobic membrane core. As noted by Miyazawa et al. (267), the extracellular spaces between the splayed helices appear to afford a lateral pathway (in addition to the large central outer vestibule pathway) for ions to access the pore.

The remainder of this section attempts to relate the Miyazawa TM domain structure with an abundance of earlier information that also bears upon TM domain structure and function. However, before doing so, it is worth briefly considering three functionally based techniques that have been of particular value in defining the secondary structure of ion channel pore-lining domains.

2. Methods for probing TM domain secondary structure

A) SUBSTITUTED CYSTEINE ACCESSIBILITY METHOD. The substituted cysteine accessibility method (SCAM) was initially applied as a means of identifying the secondary structure of ion channel pore-lining domains (5, 8). The method entails introducing cysteine residues one at a time into the protein domain of interest. Cysteine reactivity is then assayed by exposure to highly soluble, sulfhydryl-specific reagents, generally methanethiosulfonate (MTS) derivatives (180). If a functional property of the channel is irreversibly modified upon exposure to such a reagent, the cysteine is assumed to be exposed at the water-accessible protein surface. If every second residue is reactive, then the secondary structure is interpreted as β-sheet (8), whereas if every third or fourth residue is exposed, the structure is interpreted as α-helical (7). This approach is now applied more widely to probe structural changes in extramembranous domains (e.g., Ref. 234). However, a drawback of applying this approach outside the pore is that a lack of functional modification does not necessarily mean that the residue has not reacted. In other words, negative results cannot be interpreted. However, this limitation is less likely to apply in the spatially restricted environment of an ion channel pore, where attachment of a large side chain is more likely to affect current flow, thus providing a generally more reliable measure of cysteine reactivity. Various extensions to this technique have also proven useful. For example, by determining changes in cysteine reactivity in various functional states (e.g., closed, open, and desensitized), it may be possible to draw conclusions about state-dependent structural changes. Similarly, by comparing state-dependent reaction rates of positively and negatively charged reagents, it may be possible to estimate the local electrostatic potential (289, 405). The originators of SCAM have provided an excellent review of its capabilities and limitations (180).

B) TRYPTOPHAN SCANNING MUTAGENESIS. This approach involves introducing tryptophan residues one at a time into the domain of interest. Because tryptophan side chains are bulky, it is assumed that if they protrude into the relatively fluid lipid bilayer they should be less likely to disrupt receptor structure and function than if they protrude towards the protein interior (71). Experimentally, one or more basic functional properties (e.g., agonist EC_{50}) of each mutant receptor is measured, and then a correlation is drawn between the position of the introduced tryptophan and the severity of the functional consequence. As with SCAM, any resulting periodicity is interpreted as β-sheet or α-helix.

C) HYDROPHOBIC REAGENT REACTIVITY. In this approach, employed extensively by Blanton and colleagues in the nAChR (22, 40, 41), labeled hydrophobic reagents are incubated with the receptor. The identity of the residues that are covalently modified by these compounds is then determined using biochemical assays. Residues thus identified are assumed to be exposed to the lipid bilayer. Again, any resulting periodicity is interpreted in terms of secondary structure.

3. TM1

By connecting directly with the NH_{2}-terminal domain, TM1 is ideally located to act as a linkage between the ligand-binding site and the channel activation gate. Hence, an unequivocal understanding of its structure and relationship with TM2 is essential. The Torpedo nAChR crystal structure identifies TM1 as an α-helix that is initiated at the residue corresponding to Y222 of the α1-GlyR and enters the membrane at around M227. As stated above, it is likely that water-filled space surrounds the extracellular portion of this helix. In support of this, an aqueous tyrosine-specific reagent labeled two tyrosines (Y222, Y228) in TM1 of the α1-GlyR (222). Furthermore, SCAM analysis on the nAChR revealed several extramembranous TM1 residues that are accessible to modification by hydrophilic reagents (432). Several lines of evidence implicate the extramembranous TM1 residues in LGIC gating (e.g., Refs. 6, 38, 102, 363, 432). Throughout the membrane-embedded portion of the nAChR TM1 there
appears to be a distinct absence of van der Waals contacts with TM2, implying that a water-filled pocket separates the respective domains (267). However, by homology with nAChR, there may be a hydrophobic bond linking I234 (or L237) and M12 in TM2 of the α1-GlyR. At its intracellular end, the TM1 α-helix is probably terminated by the aspartic acid at position 247.

4. TM2

Affinity labeling experiments employing pore-blocking substances first suggested an α-helical open state structure of the nAChR TM2 (reviewed in Ref. 18). The Torpedo nAChR structure of Miyazawa et al. (267) confirms the long-held view that this domain forms an α-helix throughout its entire length (267). As summarized in Figure 1A, an α-helical structure is also strongly supported by SCAM analysis. Indeed, the luminal exposure patterns as determined by SCAM and the Miyazawa structure are entirely in agreement. SCAM also reveals a highly conserved pattern of residue exposure in the nAChR, GABA_R, and 5HT3R (Fig. 1A), suggesting that a similar pattern applies to all LGIC members.

To facilitate comparison between different LGIC members, a common TM2 numbering system is used (265). This system assigns position 1’ to the putative cytoplasmic end of TM2 and 19’ to the outermost residue (Fig. 1A). (Note that these assignments are confirmed by the Miyazawa TM domain structure.) A complete SCAM analysis of the GlyR TM2 is yet to be published. However, experiments conducted to date indicate the following GlyR α1-subunit residues line the pore: G2’, T6’, R19’, and A20’ (234, 341). By homology with other LGICs (7, 234, 341, 409), the following residues are also likely to line the pore: T7’, L9’, T10’, T13’, S16’ and G17’ (Fig. 1A). When viewed on an α-helical net, these residues form a hydrophilic “strip” along one side of an otherwise hydrophobic α-helix (Fig. 1B). A predicted cross-section through the α1-GlyR pore is shown in Figure 1C. The pore exposure pattern of residues intracellular to G2’ cannot currently be predicted for anionic LGICs because, as discussed in detail in section vB, they contain an additional proline at position –2’ that is likely to induce structural disruptions around the internal pore boundary. Because the GlyR β-subunit TM2 has an unusually low sequence homology with all other LGIC TM2 domains (Fig. 1A), it will be of interest to determine whether it also shares the consensus residue exposure pattern.

Structural analysis shows TM2 to be kinked radially inwards, attaining a minimum pore diameter at its midpoint (267). Miyazawa et al. (267) propose that this constriction facilitates a tight hydrophobic coupling between TM2 residues of neighboring subunits at two levels in the central region of the pore. The first contact is thought to occur between L9’ of one subunit and the 10’ residue of the adjacent subunit. In all GlyR α-subunits the 10’ residue is a threonine, but in the β-subunit it is a serine. The second intersubunit contact occurs between residues homologous to Q14’ and T13’ in neighboring TM2s of the GlyR α1-subunit (or E14’ and S13’ in the β-subunit).

Although the 19’ position defines the external border of membrane-embedded portion TM2, the α-helical structure extends into extracellular space for another 2.5 turns before terminating at the residue corresponding to V280 in the α1-GlyR (267). SCAM analyses on the GlyR and

**FIG. 1.** Pore-lining residues in the α1 glycine receptor (GlyR). A: sequence alignments of the TM2 domains of indicated LGIC subunits. Positively charged residues are shaded in blue, and negatively charged residues are shaded in yellow. Note that only cationic LGICs have a negatively charged residue at –1’. Circles denote pore-lining residues as identified by SCAM analysis (7, 234, 341, 409) or by cryoelectron microscopic analysis in the nicotinic acetylcholine receptor (nAChR) (267). In the case of the serotonin (5-HT3) receptor, dark circles denote those residues identified as pore-lining by both Refs. 288 and 316, whereas light circles denote residues identified as pore-lining by Ref. 316 only. Additional GlyR α1-subunit residues that are assumed by structural homology with other LGICs to line the pore are identified by squares. B: α-helical net representation of GlyR α1-residues with the putative pore-lining residues denoted by a white background. C: hypothetical cross-sectional view through the α1 GlyR pore. Pore-lining residues are indicated by the white backgrounds. The exposure pattern of residues deeper than G2’ cannot currently be modeled.
GABA<sub>A</sub>R confirm that most extramembranous TM2 residues have extensive contact with water (37, 234). In fact, the SCAM analysis on the GABA<sub>A</sub>R even predicted an α-helical structure for these residues (37). The TM2-TM3 linker is formed by the α1-subunit residues, V280 to D284.

The roles of TM2 in forming the channel gate, in controlling ionic selectivity, and in forming the binding sites for agents of physiological and pharmacological importance are considered in sections IV and VI.

5. TM3

According to the Miyazawa TM domain structure, the α1-GlyR TM3 α-helical domain starts at I285, with the membrane-embedded portion extending from A288 to H311 (267). There is strong support from functionally based techniques that at least the external (NH<sub>2</sub>-terminal) half of this domain forms an amphipathic α-helix. For example, evidence from the nAChR using lipophilic probes identified an α-helical like periodicity in the lipid-facing residues (40). A tryptophan scanning analysis identified an identical periodicity in the same set of residues (76). In addition, SCAM analysis of the GABA<sub>A</sub>R using water-soluble reagents also supported an α-helical periodicity in the outer half of TM3 (400, 401). A satisfying aspect of these studies was that the water-facing residues were generally displaced by one from the lipid-facing residues (see Ref. 223 for review). Consistent with structural predictions (267), the SCAM results strongly suggest that this portion of the domain contributes to the lining of a water-filled pocket distinct from the channel pore. Recent SCAM studies on the GABA<sub>A</sub>R in the absence and presence of pharmacological agents suggest that this pocket changes conformation as the channel gates (400–402). An abundance of evidence from both the GABA<sub>A</sub>R and GlyR, reviewed in section VI, provide a strong case that residues in this pocket form binding sites for alcohols and volatile anesthetics. The Miyazawa TM domain structure reveals that residues from TM3 are closely apposed to residues from both TM2 and TM4 at several points throughout their lengths (267). However, TM3 appears to contact TM1 only towards the intracellular membrane surface.

6. TM4

In addition to direct structural evidence (267), several lines of functional evidence imply that this domain forms an α-helix throughout its entire length. First, the pattern of lipid-exposed residues is consistent with an α-helical periodicity as determined by both hydrophobic probes in the nAChR (40, 41) and tryptophan scanning mutagenesis in the GABA<sub>A</sub>R (171). Second, proteolytic studies on GlyR α1-homomers did not identify cleavages in membrane-associated fragments of this domain (221), a result that is also consistent with an α-helical structure.

By structural homology with the nAChR, the α1-GlyR TM4 is likely to be initiated at K385 and terminated at V418, with the intramembranous portion extending from K389 to I408 (267). Thus the α-helix extends about 2.5 turns beyond the external membrane boundary. Although TM4 is closely apposed to TM3 throughout its length, its contact TM1 appears confined to its intracellular half (267).

C. NH<sub>2</sub>-Terminal Ligand-Binding Domain

1. Structural homology with AChBP

The fresh water snail, <i>Lymnaea stagnalis</i>, produces and stores a soluble AChBP in glial cells located near to cholinergic synapses. When released by acetylcholine stimulation, AChBP buffers the acetylcholine in the synaptic cleft (350). This protein forms a stable homopentamer and binds acetylcholine, δ-tubocurarine, and α-bungarotoxin with much the same affinity as does the α7-homomeric nAChR (350). AChBP comprises 210 amino acids and, although it lacks the TM domains, it provides a full-length model of the NH<sub>2</sub>-terminal ligand-binding domain of LGICs. It also incorporates the signature cysteine loop that is a unique feature of the LGIC family. It shares a 20–24% amino acid sequence homology with nAChR subunits and a 17% homology with the GlyR α1 subunit (Fig. 2). The crystal structure of this protein (52) represents a major breakthrough in our understanding of LGIC structure and function. Due to both its significant sequence homology and to its functional similarity with the α7-homomeric nAChR, its structure is considered an accurate template of the NH<sub>2</sub>-terminal ligand-binding domain of the nAChR and, by inference, of other LGIC members.

In three dimensions, AChBP forms a hollow cylinder with an external diameter of 80 Å, a height of 62 Å, and an inside diameter of 18 Å. Its size and general shape are in good agreement with that previously determined from electron diffraction images of Torpedo nAChRs (266). A model of the GlyR α1-subunit ligand-binding domain based on the AChBP structure is shown in Figure 3. Each of the five subunits is positioned in a radially symmetrical manner around the central pore. When viewed from above (i.e., from the synapse, looking towards the membrane), the protein is said to resemble “a 5-bladed windmill toy” (52). Individual subunits contain an α-helix near the NH<sub>2</sub>-terminal extremity and then a series of 10 β-sheets with short 3<sub>10</sub> helices following the second and third β-sheets. The β-sheets 1–7 form a “twisted β-sandwich” with β-sheets 8–10, resulting in 2 separate hydrophobic cores. Together the β-sheets form a modified immunoglobulin fold. Pockets are present at the subunit interfaces, approximately midway between the top and
bottom of the protein, and abundant evidence (reviewed in Refs. 74, 179) identifies these as ligand binding sites. This pocket is lined by three loops from one subunit that form the “principal” (or +) side of the ligand-binding pocket, whereas three β-sheets from the adjacent subunit form the “complementary” (or −) side of the pocket. Viewed from the top of the complex, the complementary side of each AChBP binding site is situated anticlockwise relative to the principal side (52). The AChBP binding site opens to the outside of the complex and, unlike the Torpedo nAChR electron diffraction images (266), there is no entry to the binding site from the central pore side of the protein.

The AChBP structure reconciles many years of biochemical and electrophysiological investigations into the structure and function of the nAChR. As discussed later in sections V and VI, it also reconciles an accumulation of structure-function data from the GlyR. In particular, it
provides an excellent basis for understanding the glycine and strychnine binding sites and the zinc inhibitory site.

It was recently proposed that sections of the α1-GlyR NH₂-terminal domain between residues 158–165 and 181–191 may be associated with the plasma membrane (223). Because the AChBP domain corresponding to 158–165 is located at a subunit interface well away from the membrane, a direct membrane interaction seems unlikely. However, residues 181–191 indeed lie toward the lowest point of the structure, between β-sheets 8 and 9, and thus could conceivably dip into the membrane.

Apart from the direct polypeptide chain linkage between β-sheet 10 and TM1, structural and functional evidence suggest 2 likely points of contact between the ligand-binding domain and the transmembrane domain. These regions are the conserved cysteine loop and the loop linking β-sheets 1 and 2 of AChBP. This later loop is also known as “loop 2.” Both loops have been proposed to
interact closely with the TM2-TM3 linker domain (181, 267), and the nature of these proposed interactions is considered in more detail in section V.D.

2. Glycosylation

As shown in Figure 2, the GlyR α1-subunit contains a glycosylation consensus site at N38, with other α-subunits containing similar sites at the homologous positions. The GlyR α2-subunit contains additional consensus sites at N45 and N76 (199). On the other hand, the β-subunit contains consensus sites at N33 and N220 (137). The first suggestion that the α1-subunit may be glycosylated was the finding that mutations to N38 prevented surface expression of functional α1-GlyRs (10, 200). Recently, it was found that glycosylation of α1-subunits is a necessary prerequisite for homomeric receptor assembly and that receptor assembly is required for transit from the endoplasmic reticulum to the Golgi apparatus and subsequently to the cell membrane (140). The question of whether β-subunits are glycosylated remains to be addressed.

D. Large Intracellular Domain

As the large TM3-TM4 domain is poorly conserved among LGIC members, both in terms of its length and amino acid sequence, it is likely to exhibit considerable structural variation as well. The only structural information to date suggests that the Torpedo nAChR intracellular domains form a hanging gondola-type structure with transverse holes (or "portals") connecting the pore with the cytoplasm (266). Because these portals are approximately the same size as a permeating ion plus its first hydration shell (266), they are ideally suited to influence ion permeation. Indeed, it has recently been shown that the deletion of three positively charged residues in the 5-HT3AR TM3-TM4 domain dramatically increases the pore unitary cation conductance (183), implying that these residues may frame the portals. The homologous region of the GlyR α1-subunit is denoted by gray shading in Figure 2. The GlyR β-subunit has an unusually large internal domain, comprising 130 residues, whereas the α1-subunit contains 86 residues (Fig. 2). The intracellular domains of both α- and β-subunits contain a variety of sites that mediate interactions between the GlyR and cytoplasmic factors. These putative interaction sites are now considered.

1. Ubiquitination domain

Under appropriate conditions, intracellular ubiquitin molecules can covalently attach themselves to specific lysine side chains on the cytoplasmic protein surface. In fact, multiple ubiquitin molecules can attach themselves end to end in a piggyback manner, resulting in a condition termed “polyubiquitination.” Ubiquitination or polyubiquitination precipitates the internalization and degradation of many protein types, including surface-expressed α1-GlyRs (55). Following internalization, the ubiquitin molecules induce the 49-kDa GlyR α1-subunit to be proteolytically nicked into a glycosylated (i.e., NH2-terminal) 35-kDa fragment and a 17-kDa COOH-terminal fragment. These fragment sizes are consistent with the ubiquitination domain lying in the large intracellular domain. The TM3-TM4 domain contains a total of 10 lysine residues (Fig. 2), several of which probably need to be individually ubiquitinated before GlyRs can be endocytosed (55). This mechanism is likely to be important in regulating the number of surface-expressed GlyRs per postsynaptic density.

2. SH3-binding motif

Because prolines induce kinks into peptide chains, regular spacing of these residues can form helical structures known as polyproline (P II) helices. Circular dichroism studies reveal the GlyR α1-subunit to contain a significant fraction (9%) of this structure (58). A certain class of protein-protein interaction sites, termed SH3 domains, are formed from PII helices (290). As recently noted (58), GlyR α1- and β-subunits both contain SH3 consensus sequences in their large intracellular domains (Fig. 2). Although the role of these domains has yet to be investigated, they may be involved in GlyR trafficking or cytoskeletal attachment.

3. Phosphorylation sites

The locations of phosphorylation consensus sites in the α1- and β-subunits are shown in Figure 2. The evidence that phosphorylation of these sites is able to modulate GlyR function is considered in section VI.A.

4. Gephyrin binding domain

This important molecule has long been known to copurify with the native GlyR as a 93-kDa protein (300). Kirsch and Betz (188) showed that it mediates the clustering of GlyRs at postsynaptic sites. Gephyrin interacts with a large and growing number of binding partners, suggestive of a high degree of complexity in the regulation of GlyR clustering. A description of the interactions of gephyrin with molecules other than the GlyR is beyond the scope of this review. Developments in this area are moving rapidly, and recent progress has been covered in several excellent reviews (189, 190, 219). The GlyR gephyrin contact site was isolated to an 18-amino acid domain in the central region of the β-subunit TM3-TM4 loop (259) (Fig. 2). Insertion of the gephyrin binding domain into the α1-subunit promotes the clustering of α1-homomeric
GlyRs (220, 259). A site-directed mutagenesis study isolated gephyrin binding activity to multiple hydrophobic residues in this domain (191). A hydropathy plot suggests that this region forms an irregular amphipathic helix, with the putative gephyrin-binding residues located along the hydrophobic side.

5. Basic cluster required for TM3 integration

A positively charged cluster, RFRRKRR, located close to the intracellular boundary of TM3 in the α1-subunit (Fig. 2), appears to be important for the correct membrane insertion of TM3. It was found that neutralization of positive charges in this cluster prevented the correct translocation of TM3-TM4 into the lumen of the endoplasmic reticulum (328). This effect was rectified by deleting positive charges from TM2-TM3. From these results, it was concluded that the basic cluster is necessary to compensate for the positively charged residues in TM2-TM3 which would otherwise preclude the correct membrane insertion of TM3 (328).

E. Receptor Assembly

1. Subunit stoichiometry and arrangement

The subunit composition of the GlyR was determined by cross-linking its polypeptides with cross-linking reagents of various specificities and lengths (209). Because the size of the largest cross-linked product totaled approximately five times the mean individual subunit size, functional membrane GlyRs were concluded to comprise pentamers. Of course, since a pentameric subunit arrangement was well-established in other LGIC members (and now in AChBP), it is scarcely conceivable that the GlyR quaternary structure would have been different. Oddly, however, for a trimeric α1-homomeric subunit composition has recently been deduced on the basis of both laser scattering and single particle electron microscopic analyses (413). A substantial measure of credibility must be granted to this study as the same group also proposed a pentameric GABA<sub>A</sub>R structure using the same techniques. These are the only GlyR images published to date, and further investigation into the basis of these findings appears warranted. One possibility is that the structure is distorted by a closely associated protein.

Although GlyRs almost certainly comprise pentameric subunit complexes, the stoichiometry and subunit arrangement of heteromeric receptors are less certain. An invariant 3α:2β stoichiometry was proposed based on the observation that α-subunits predominated over β-subunits in all tissues examined (209). Although no more direct evidence in favor of any particular stoichiometry has ever been presented, the 3α:2β ratio is a long-held dogma in the field. Even if this stoichiometry is correct, there is no compelling rationale for distinguishing a side-by-side β-subunit arrangement from one whereby the β-subunits are separated by an α-subunit. Knowledge of this is particularly important for understanding GlyR molecular pharmacology because binding sites of all types are located at subunit interfaces (74), and the number of α-α, α-β, β-α, and β-β interfaces per receptor cannot currently be determined. A careful reanalysis of subunit stoichiometry and arrangement in αβ-heteromeric GlyRs is overdue.

2. Intersubunit contact points and assembly domains

GlyR α1- and α2-subunits appear to be able to coassemble in a random binomial manner that is dependent only on the relative abundance of each subunit (200). However, when β-subunits are present, the αβ subunit stoichiometry appears to be invariant, as inferred from the monotonic nature of the glycine dose-response (200). The regions of the GlyR β-subunit responsible for this behavior have been investigated in detail (140, 200). The initial characterization involved coexpressing α1-subunits with chimeric subunits made from α1- and β-subunits. Fixed assembly was found to require the extracellular, but not the TM or intracellular regions, of the β-subunit (200). To further delineate the regions responsible for subunit assembly, certain divergent domains (termed “assembly boxes”) in the β-subunit NH<sub>2</sub>-terminal domain were mutated back towards the α1-subunit sequences to see whether they permitted the individual chimeric subunits to express as homomers. Indeed, the introduction of various combinations of boxes permitted homomeric assembly (200). The important boxes involved in this process are shown in Figure 2. As a minimum requirement, box 1 has to combine with either box 3 or box 2 plus box 8 to result in α1-homomer formation (140, 200). The critical α1-subunit residues that must be set towards the α1-subunit sequence are as follows: box 1 (P35, N38, S40) and box 3 (L90, S92), or box 2 (P79) and box 8 (N125, Y128) (140). Note that N38 is a glycosylation site. The intersubunit contact points between AChBP subunits and their predicted counterparts in the GlyR α1-subunit are shown in Figure 2. It is apparent that the boxes do not directly form the interfaces, although boxes 3 and 8 lie directly adjacent to interface sites. This suggests that the box mutations act allosterically to modulate the interface conformation. The roles of the interface residues themselves in controlling subunit assembly have yet to be investigated.

3. Effects of high receptor density

The injection of increasing amounts of α1-subunit cDNA into Xenopus oocytes results in a progressive increase in both maximum current (I<sub>max</sub>) and glycine sen-
sitivity (90, 362). A recent study has provided a possible explanation for this. When α1-GlyRs containing introduced gephyrin binding domains were clustered using gephyrin, they exhibited an additional extremely fast desensitizing current component (220). Although the glycine EC50 of the peak current was similar to that of unclustered receptors, the EC50 of the plateau current was significantly reduced. Fast (submillisecond) solution application to small (HEK293) cells was required to see the fast desensitizing component. Since such rapid solution exchange is difficult to achieve with Xenopus oocytes, it seems possible that the fast desensitizing component was missed in the earlier study. These effects could be the result of direct interactions between adjacent receptors, allosteric actions of gephyrin on the α-subunit, or depletion of a cytoplasmic cofactor necessary for normal receptor function.

4. Coassembly with other LGIC subunits

Functional evidence suggests that the GABA<sub>C</sub> ρ1-subunit can coassemble with glycine α1- and α2-subunits in vitro (287). This study showed that the recombinant expression of a mutant ρ1-subunit with the α1- or α2-subunits caused a change in the gating of GABA-induced currents. Because homomeric GlyRs were not activated by GABA, it was proposed that the change in pharmacology must have been due to coassembly of ρ1- with α-GlyR subunits. This finding raises the intriguing possibility that such heteromeric receptors might also exist in vivo.

IV. STRUCTURE AND FUNCTION OF THE PORE

A. Functional Properties of the Pore

1. Ionic selectivity

Although GlyRs are strongly selective for anions over cations, they have a small but measurable permeability to K<sup>+</sup> and Na<sup>+</sup> (45, 186). Native GlyRs expressed in cultured neurons have a permeability sequence of SCN<sup>−</sup> > NO<sub>3</sub> <sup>−</sup> > I<sup>−</sup> > Br<sup>−</sup> > Cl<sup>−</sup> > F<sup>−</sup> (45, 107). This sequence is in proportion to the ionic hydration energies, implying that the removal of waters of hydration is the major barrier to ion channel entry. Because electrostatic interactions with pore sites are, by inference, less important, this sequence corresponds to a “weak field strength” binding site. The GlyR pore also exhibits anomalous mole-fraction behavior, suggesting the presence of at least two interacting binding sites (45, 107). The permeability has also been probed with large organic anions. From space-filling models of the molecules tested, the narrowest part of the pore is estimated to have a diameter of at least 5.2 Å in spinal neuron GlyRs (45), 5.5–6.0 Å in hippocampal neuron GlyRs (107) and 5.22–5.45 Å in recombinant GlyRs (324).

By comparison, cationic LGICs also possess a weak field strength binding site, but they have not been shown to display anomalous mole fraction effects and have a significantly larger minimum pore diameter of at least 7.5 Å (225).

2. Single-channel conductance

GlyRs display multiple unitary conductance states (45, 46). A useful comparison of GlyR conductance states in native neuronal GlyRs, as well as in various recombinant subunit configurations, is presented in Table 1 of Reference 307. Recombinant α1-GlyRs exhibit five conductance states ranging between 20 and 90 pS, with the 90-pS state occurring with the greatest frequency. The α2- and α3-GlyRs share the same conductance states but also exhibit a frequently visited 110-pS conductance level. This characteristic is conferred to the α1-GlyR by the G2′A (α1 → α2) mutation (46). Coexpression of α1-subunits with the β-subunit eliminates the highest conducting levels, leaving a 45-pS state as the most frequently occurring state (46). Incorporation of the E20′/S (β → α1) mutation into the β-subunit confers α1-GlyR-like conductance levels to heteromeric GlyRs. Because neither mutation abolishes the preexisting conductance states, it is difficult to determine whether their actions are mediated allosterically or via direct interactions with permeating ions. The relative probabilities of entering the predominant conductance states do not vary with agonist concentration (25, 374).

As mentioned above, elimination of a series of three positively charged residues in the 5-HT<sub>3</sub>AR TM3-TM4 domain resulted in a dramatic increase in the single-channel conductance (183). It was proposed these residues might line a narrow portal that links the pore with the cytosol. It is yet to be established whether the homologous residues in the GlyR α- or β-subunits influence the unitary chloride conductance. However, since both positively and negatively charged residues line the corresponding domain in the GlyR α1-subunit (Fig. 2), and the precise alignment is unknown, it is difficult to predict what this influence might be.

B. Molecular Determinants of Ion Selectivity and Conductance

Much of the groundwork for our current understanding of GlyR permeation mechanisms has come from studies on the nAChR. One classic experiment showed that the open-channel blocker QX-222 reached as far as the 6′ position when applied externally in the open state (61, 224), implying that the most constricted section of the pore was located more internally than this point. Because residues in the narrowest part of the pore are more likely to influence both unitary conductance and ion selectivity,
this in turn implied that the residues near the intracellular boundary of TM2 controlled both processes. A recent SCAM study has provided the most definitive functional evidence to date for a drastic pore constriction between the $-2'$ to the $+2'$ positions (404). It should be noted, however, that the Miyazawa TM domain structure shows the pore widening considerably from the $+2'$ to $-2'$ positions (267). The reason for this apparent mismatch is not yet understood.

Charged residues are obvious candidates as ionic selectivity sites. As shown in Figure 1A, the nAChR $\alpha$-subunit contains four charged residues in the vicinity of TM2: a negatively charged aspartic acid at $-4'$ (the “cytoplasmic ring”), negatively charged glutamic acids at $-1'$ (the “intermediate ring”), and 20' (the “outer ring”) and a positively charged lysine at 0'. Three lines of evidence indicate that K0' faces away from the pore: 1) charge-reversal mutations have no effect on single-channel conductance (168), 2) the pore has a strong negative electrostatic potential in this region (289), and 3) the Miyazawa TM domain structure unequivocally shows K0' facing away from the pore (267). Although charge-reversal mutations to the cytoplasmic and outer rings significantly affect single-channel conductance (168, 169), neither site appears to significantly impede the access of negatively charged molecules to deeper regions of the pore (404). However, mutations to the intermediate ring strongly influence both single-channel conductance (168) and pore effective diameter (387). In addition, mutations to 2' residues also affect cation selectivity and effective diameter (reviewed in Ref. 225). Thus residues at the $-1'$ and $+2'$ positions, which are separated by one $\alpha$-helical turn in the narrowest part of the pore, form the main selectivity filter. Structural predictions indicate that both side chains have extensive exposure to the pore (267).

By comparing TM2 sequences between the $\alpha7$-nAChR and the $\alpha1$-GlyR, Galzi et al. (126) sought to determine the minimum number of GlyR residues required to switch the nAChR pore from cation selective to anion selective. The minimum requirement was found to be the E-1'A and V13'T mutations as well as the insertion of a proline between $-2'$ and $-1'$ (i.e., $-2'$). The same mutations achieved a similar effect in the 5-HT$_3$R (149). Because the E-1'A and V13'T mutations alone do not convert selectivity, they are considered to be “permissive” rather than “essential” for anion permeability (73). Interestingly, the proline insertion site was not critical: selectivity reversal was also effected by inserting it into the $-4'$ position (73). Together these results imply that selectivity reversal required a change in either the lumen geometry or in the exposure profile of side chains lining the selectivity filter.

The reverse triple mutation (A-1'E, T13'V and deletion of $-2'$) was subsequently found to convert $\alpha1$-GlyR selectivity from anionic to cationic (185). However, the resultant channels had a low conductance (3 pS at $-60$ mV) and converted the ratio of permeabilities ($P_{Cl}/P_{Na}$) from 24 to 0.3, implying that Cl$^-$ permeation may have been reduced without a concomitant increase in Na$^+$ permeation. A stronger case for an increase in cation permeability was made by the same group when they showed that the reverse double mutation (A-1'E and deletion of $-2'$P) decreased the $P_{Cl}/P_{Na}$ further to 0.13, while increasing the unitary cation conductance to 7 pS at $-60$ mV (186, 272). Furthermore, these mutations increased the effective pore diameter of this GlyR to approximately the value seen in the nAChR. Curiously, however, selectivity reversal was also achieved by incorporating only the A-1'E mutation into both the $\alpha1$-GlyR and the $\rho1$-GABA$_\text{A}$R (186, 406). From this result, it is tempting to speculate that anion selectivity is associated with a net positive electrostatic charge caused by both the elimination of the negative charge at E-1' and an enhanced pore exposure of R0'..

However, one problem with the “net positive charge” model of anion permeation is that the anion selectivity of the triple mutant $\alpha7$-nAChR is not affected by the charge-eliminating K0'Q mutation (73). In addition, the E-1'A mutation alone did not increase anion permeability (73). Indeed, these two observations prompted Corringer et al. (73) to suggest that the anion-selective nAChR pore may be lined by polar groups from the peptide backbone. Further insight into this issue was recently provided by the finding that the $\rho1$-GABA$_\text{A}$R anion-to-cation selectivity switch was conferred by the charge-reversing mutation R0'E, but not by the charge-eliminating mutations R0'C or R0'M (406). It appears feasible to reconcile all these findings by proposing that a net negative charge in the $-1'$ region is associated with cation selectivity, whereas a net neutral or positive charge is associated with anion selectivity. In the event of a neutral potential in this region, positive dipoles of local polar groups may confer anion permeability. A critical test of this proposal would be to quantitate the GlyR pore electrostatic potential profile using SCAM (289, 405). It would also be informative to investigate the effects of a variety of substitutions at the $-1'$ and 0' positions to differentiate the effects of side chain charge, size, hydrophilicity, and hydrophobicity on ion selectivity and pore effective diameter. Unfortunately, however, mutations to these residues have a habit of precluding functional receptor expression.

The influence of $\beta$-subunits on ion permeation has yet to be investigated in detail. As shown in Figure 1A, $\alpha$- and $\beta$-subunits have a low sequence homology throughout TM2. Of particular note, the $\beta$-subunit includes an alanine-for-proline substitution at $-2'$ and a proline-for-glycine substitution at position 2'. These substitutions suggest the $\beta$-subunit secondary structure may be different from the pore selectivity filter. However, as summarized
above, experiments to date suggest the \(\beta\)-subunit does not induce drastic changes in permeation characteristics.

The effect on permeation of the outer ring of charge, formed by R19', has also been investigated in the \(\alpha_1\)-GlyR. Elimination of this charge by the human startle disease mutations R19'L and R19'Q caused a decrease in the unitary \(\text{Cl}^-\) conductance (208, 305). More recently, it was shown that the R19'A and R19'E mutations increased the unitary cation conductance in cation-selective GlyRs and changed the rectification properties of the pore (272). Together, these results are consistent with an electrostatic contribution of R19' to ion permeation. However, R19' does not preclude the entry of positively charged molecules into the GlyR pore (341, 343) and is more likely to affect conductance by concentrating anions in the outer vestibule (186). The \(\beta\)-subunit E20'S mutation, which neutralizes a negative charge at the adjacent position, may also increase the single-channel conductance via an electrostatic mechanism (46).

An impressive array of \(\alpha_1\)-GlyR pore functional properties was reconciled by a three-dimensional Brownian dynamics simulation study (284). This study used a model of the pore based on the TM2 primary structure and the best available estimates of pore diameter prior to publication of the Miyazawa study (267). The selectivity filter diameter was permitted to vary between 6 and 8.3 \(\AA\), but the charge at R0' was held constant at +0.375e. Under these conditions, the first \(\text{Cl}^-\) experiences a deep energy minimum near R0'. A second \(\text{Cl}^-\) entering the pore experiences a weaker energy minimum near the pore midpoint. These two ions exist in equilibrium. A third entering \(\text{Cl}^-\) abolishes these energy wells, allowing the innermost ion to escape into the intracellular solution. In light of the preceding discussion, it would be interesting to determine the effects on permeation of variations to the charge at R0'.

C. The Channel Activation Gate

This gate refers to the physical barrier that stops ions from traversing the pore in the unliganded state. There is no information available to date as to its location in the GlyR. There are, however, two schools of thought as to its location in other LGIC members. One proposal, supported by the structural analyses of Unwin, Miyazawa, and colleagues (266, 267, 376), is that the TM2 domains are kinked inwards to form a centrally located gate near the highly conserved L9' residue. However, the results of SCAM experiments on the GABA\(_A\)R and nAChR are inconsistent with the gate lying more externally than the 2' position (7, 289, 409). A particularly detailed study on the nAChR, which probed the accessibility of cysteines introduced into TM2 to both sides of the membrane in the closed and open states, delimitated the gate to the same narrow pore region (−2' to +2') that houses the selectivity filter (404).

D. Molecular Mechanisms of Desensitization

Desensitization is a property whereby an agonist-gated channel closes in the continued presence of agonist. In general, the rates of onset and recovery from desensitization are important parameters governing the size, decay rate, and frequency of fast synaptic currents (175). Until recently, it was considered that GlyRs desensitized too slowly to influence these parameters (219). However, a recent study using ultra-fast solution exchange identified a very rapid desensitization component (decay time constant \(\approx 5\) ms) that is induced by either the clustering of \(\alpha_1\)-GlyRs (220) or by coexpression of the \(\alpha_1\)- with the \(\beta\)-subunit (268). This rapid component could conceivably influence the properties of repetitive glycineergic synaptic currents. However, further research is required to clarify the role, if any, of GlyR desensitization at glycineergic synapses.

The location of the desensitization gate in the GlyR is not yet known. However, SCAM evidence from the nAChR suggests that the desensitized state is associated with a crimping of the channel pore between the −2' and 9' residues (403). In contrast, a similar approach showed that the activation gate crimps the pore between only the −2' and +2' positions.

A growing body of evidence reveals that intracellular domains can profoundly influence GlyR desensitization. A functional comparison of human \(\alpha_3\)- and \(\alpha_3\)-GlyRs showed that the removal of a 15-amino acid segment from the large intracellular domain dramatically increased the desensitization rate (280). Desensitization rate is also dramatically enhanced by the human startle disease mutations, I244N and P250T (also known as P-2'T), in the \(\alpha_1\)-GlyR TM1-TM2 loop (237, 334). Other mutations in this region, including W243A, I244A, and A251E (also known as A-1'E), have similar effects (186, 237). The relationship between desensitization rate and the properties of side chains introduced into the P250 position showed that bulky hydrophobic residues yielded the fastest desensitization rates (51). Thus desensitization rate is exquisitely sensitive to structural perturbations in the TM1-TM2 loop. However, in the GlyR at least, such observations are of phenomenological interest only until it can be demonstrated that events that alter desensitization in vivo do so by varying the conformation of this domain.

V. AGONIST BINDING AND RECEPTOR ACTIVATION

A. Introduction

This section considers the molecular basis by which agonists bind to and activate (or gate) the GlyR. In particular, it will consider the following important questions.
What is the structure of the binding site and where is it located? What is the molecular basis for binding site selectivity? What structural changes underlie the activation of the receptor? One problem with addressing these questions is that it is difficult to experimentally dissect binding from gating mechanisms as the two processes are tightly coupled (72). To understand how this separation may be achieved, it is necessary to briefly consider the theory of receptor activation.

1. Review of basic receptor theory

In its simplest form, the receptor activation process can be represented as:

\[
K_A \frac{E}{A + R} \leftrightarrow AR \leftrightarrow AR^*
\]

where \( A \) is the agonist, \( R \) is the vacant receptor, \( AR \) is occupied but shut, and \( AR^* \) is occupied and activated. The equilibrium constant for binding (or binding affinity) is given by:

\[
K_A = \frac{[A][R][AR]}{k_{doff}} = k_{off}/k_{on}
\]  

where \( k_{doff} \) is the dissociation rate constant (units of \( s^{-1} \)) and \( k_{on} \) is the association rate constant (units of \( M^{-1}s^{-1} \)). The equilibrium constant for gating (or efficacy) is given by:

\[
E = \frac{[AR^*][AR]}{[A][R]} = \frac{\beta}{\alpha}
\]

where \( \beta \) is the opening rate constant and \( \alpha \) is the closing rate constant (both with units of \( s^{-1} \)). With the use of classical receptor theory (along with some simplifying assumptions), it can be shown (72) that the agonist concentration required for a half-maximal response:

\[
EC_{50} = K_A/(1 + E)
\]

and the maximum fraction of receptors in the activated state (or maximum open probability)

\[
Po_{max} = E/(1 + E)
\]

Equation 3 tells us that the \( EC_{50} \) is a function of both the binding and gating properties of the receptor. Equation 4 tells us that variations in \( E \) have no measurable effect on maximum open probability unless they occur within a limited range of \(-0.1\) to \(10\).

The structural basis of GlyR binding and gating has mainly been investigated using site-directed mutagenesis coupled with functional (i.e., electrophysiological) analysis. If a mutation affects mainly \( K_A \), then it is assumed to affect binding, either directly or allosterically. If a mutation affects mainly \( E \), then the mutated residue is assumed to affect the conformational change leading to the open state, either directly or allosterically. Single-channel kinetic analysis can provide reasonably direct estimates of both \( K_A \) and \( E \). A more convenient, but less precise, method involves measuring relative changes in the peak whole cell current (\( I_{max} \)) activated by partial agonists. Since \( I_{max} = iPo_{max} \) where \( i \) is single-channel conductance and \( n \) is number of activated receptors, \( I_{max} \) provides a qualitative measure of changes in \( E \), assuming that \( i \), \( n \), and desensitization rate remain constant.

2. Concerted versus sequential models of receptor activation

The above model of receptor activation is an oversimplification because \( \alpha \)-GlyRs, as pentameric multimers, contain five agonist binding sites. Two starkly contrasting models have been proposed to describe multimer protein allosteric mechanisms. These are the coupled Monod-Wyman-Changeux (MWC) model (60) and the uncoupled or sequential Koshland-Nemethy-Filmer (KNF) model (193). In the simplest version of the MWC model, all the subunits change conformation simultaneously, and in consequence, the receptor can exist in only the closed or entirely activated states. In contrast, in the KNF model, each subunit can independently adopt a specific conformation change depending on the number of bound agonist molecules, leading to a series of intermediate protein conformational states. Extended MWC models that allow for multiple functional states and subunit asymmetry can explain many characteristics of nAChR behavior (60). In particular, two simple observations that support an MWC model over a KNF model are that 1) nAChR single-channel conductance is independent of agonist concentration and 2) spontaneous channel openings are occasionally seen in the absence of agonist. As discussed in the ensuing paragraphs, the information available to date also favors an MWC model for the GlyR.

B. Kinetic Models of Glycine Receptor Gating

The single-channel kinetic properties of GlyRs were first characterized in native receptors from embryonic mouse spinal neurons (374). This study revealed several important features of GlyR kinetic behavior. First, unlike nAChRs, GlyR channel openings did not occur in the absence of agonist. Second, although the single-channel conductance was not constant, it displayed no variation with agonist concentration. The third and perhaps most revealing feature was that there were at least three exponential components in the open period distributions.
Short-lived channel bursts (corresponding to the faster exponential components) predominated at low concentrations, whereas the relative frequency of longer lived bursts (slower exponential components) increased at higher concentrations. It was therefore concluded that progressively longer-lived states were associated with increasing numbers of bound glycine molecules and that maximal channel activation therefore required a minimum of three bound glycines (374). Because the molecular identity of the mouse GlyRs is not known, the number of glycine binding sites per receptor is uncertain. Irrespective of this, the three-open-state model is in broad agreement with several more recent studies on recombinant α1-GlyRs (25, 123, 139, 212, 227). However, one study that examined the equilibrium gating of recombinant α1-GlyRs identified two further burst-length components, namely, a particularly short-lived state that was prominent at low glycine concentrations and a particularly long-lived state that was seen only at the highest tested concentrations (25). The authors interpreted these results by proposing that the shortest to longest lived states correspond to receptors occupied by one to five glycine molecules, respectively. These findings were reconciled into an MWC-like model where any one of five possible liganded states can lead to channel opening. An attractive feature of this model is that α1-GlyRs do indeed possess five glycine-binding sites.

However, this model is controversial because it predicts that mono-ligated openings should add an instantaneous linear component to the onset of the glycine-stimulated response, and experiments involving the rapid application of glycine to both native and recombinant GlyRs have revealed the distinct absence of such a component (128, 139, 218, 227). The shape of the glycine activation response was consistent with a minimum of two bound glycines being required to activate the native GlyR (218) or a minimum of two or three bound glycines for activation of the recombinant α1-GlyRs (128, 139, 227). Thus the GlyR kinetic models generated by analysis of stationary and nonstationary receptor kinetics cannot be resolved at present. Because such models are crucial for understanding and predicting receptor behavior, it is hoped that both stationary and nonstationary kinetic analyses will be included in the development and functional testing of future models.

In contrast to the α1-GlyR, the α2-GlyR can be modeled as possessing only a single open state (246). It also has an extraordinarily slow rate of receptor activation that requires at least two simultaneously bound glycine molecules (246). Mangin et al. (246) successfully modeled these properties by proposing that the single open state was linked to the fully liganded closed state.

Transitions to and from desensitized states have also been modeled in α1- (128, 218, 246) and α2-GlyRs (246). These models depict these states as being accessed from the singly or doubly liganded closed states. Some kinetic studies have ignored the effects of desensitization because it generally occurs at a relatively slow rate.

C. Agonist Binding

1. Agonist affinity and efficacy

Native and recombinant GlyRs are activated by amino acid agonists with the following rank order of potency: glycine > β-alanine > taurine > GABA. In α1-GlyRs expressed in HEK293 cells, glycine, β-alanine, and taurine all behave as full agonists, with glycine exhibiting an EC₅₀ value of 20–50 μM (46, 237). However, when the same α1-GlyRs are expressed in Xenopus oocytes, the EC₅₀ values of all agonists are increased by about an order of magnitude, and the peak magnitudes of saturating β-alanine-, taurine- and GABA-gated currents are reduced relative to those activated by glycine (214). These differences, shown in diagrammatic form in Figure 4, imply variations in GlyR conformation between the two expression systems. A recent detailed study of Xenopus oocyte-expressed α1- and α2-GlyRs showed that the glycine, taurine, and GABA sensitivity varied in parallel from cell to cell over a surprisingly large (10-fold) range (90). In addition, the relative peak magnitudes of taurine- and GABA-gated currents varied according to their EC₅₀ values. The origin of this variability is not known, but such a degree of variability has not been reported in GlyRs expressed in HEK293 cells.

The information available to date suggests that α2-, α3-, and α4-GlyRs exhibit similar agonist sensitivities to the α1-GlyR (90, 130, 157, 201, 202, 246). The incorpora-
tion of \( \beta \)-subunits has little effect on receptor sensitivity to glycine (154, 298, 288), although its effect on the \( EC_{50} \) values for other agonists has not been determined to date.

A combination of rapid agonist application techniques and equilibrium single-channel kinetic analysis was used by Lewis et al. (227) to estimate the agonist affinities and efficacies of glycine, \( \beta \)-alanine, and taurine on \( \alpha 1 \)-GlyRs expressed in HEK293 cells. The respective \( K_A \) values were estimated as follows: glycine, \( \approx 160 \, \mu M \); \( \beta \)-alanine, \( \approx 360 \, \mu M \); and taurine, \( \approx 460 \, \mu M \). These differences were found to be entirely due to variations in the agonist dissociation rates. The \( E \) values were predicted to be 16, 8.4, and 3.4 for glycine, \( \beta \)-alanine, and taurine, respectively (227). The differences among these values were caused by variations in the channel opening rate, with the channel closing rate remaining constant for all three agonists. Earlier studies on homomeric \( \alpha 1 \)-GlyRs (128) and heteromeric \( \alpha 1 \beta \)-GlyRs (218) estimated comparable \( E \) values for glycine. In contrast, the homomeric \( \alpha 2 \)-GlyR was estimated to have a glycine \( E \) value of at least 250 and a \( K_A \) value near 40,000 \( \mu M \) (246).

2. Agonist binding domains

As discussed in detail in section \( \mu C \), the ligand-binding pocket is formed as a cleft between adjacent subunits. Three loops (domains A, B, and C) form the "principal" ligand-binding surface on the + side of the interface (Fig. 3A) and three \( \beta \)-strands (domains D, E, and F) from the adjacent subunit comprise the "complementary" (or −) face. The residues of AChBP that contribute to these domains are indicated in Figure 2. The human GlyR \( \alpha 1 \)- and \( \beta \)-subunit residues that align with the AChBP ligand-binding site residues are also shown.

Kuhse et al. (202) observed that the GlyR \( \alpha 2^\alpha \)-splice variant subunit had a glycine \( EC_{50} \) that was \( \approx 40 \) times higher than those of the \( \alpha 2 \)- or \( \alpha 1 \)-subunits. Glycine sensitivity was restored by incorporating the E167G mutation into the \( \alpha 2^\alpha \)-subunit, thus reversing the only nonconserved amino acid between the \( \alpha 2^\alpha \) and \( \alpha 2 \)-subunits. This residue aligns with G160 in agonist binding domain B of the GlyR \( \alpha 1 \)-subunit (Fig. 2). It was subsequently shown that the double mutant F159Y, Y161F \( \alpha 1 \)-GlyR caused a modest (12-fold) increase in glycine sensitivity, but surprisingly large (121- and 45-fold) increases in the sensitivities to \( \beta \)-alanine and taurine (338). Although \( EC_{50} \) changes alone do not provide evidence for binding interactions, domain B was considered likely to bind glycine since 1) the mutation also dramatically affected GlyR sensitivity to the competitive antagonist strychnine (see below), and 2) this domain had already been identified as an nAChR binding site (reviewed in Ref. 19). The domain has since been implicated in agonist binding in the GABA\( _A \) and 5-HT\( _3 \) receptors (14, 354).

The agonist-binding role of the GlyR \( \alpha 1 \)-subunit C domain has been investigated by the Schofield \( \alpha 1 \)-subunit C domain has been investigated by the Schofield group (309, 381, 382). The GlyR is unusual among LGICs in that its C domain is contained within a second extracellular disulfide loop. It was shown that mutations to the even-numbered residues, L200, Y202, and T204, profoundly affect receptor sensitivity to glycine or strychnine, or both (309, 381, 382). Of particular note, the conservative Y202F mutation caused a drastic increase (480-fold) in the glycine \( EC_{50} \), while having little effect on strychnine sensitivity (309). Due to the differential sensitivity of specific residues to glycine and strychnine and the fact that this domain had been implicated in acetylcholine binding to the nAChR (reviewed in Ref. 19), binding domain C is likely to be involved in glycine binding. However, most mutations to this domain also affected receptor gating, as evidenced by their conversion of taurine from a full into a partial agonist (309).

Residues I93 and N102 in domain A were identified as agonist-binding elements on the grounds that conservative mutations affected agonist \( EC_{50} \) values only, without affecting strychnine sensitivity or the agonist efficacies of \( \beta \)-alanine or taurine (151, 379). The R97G mutation, which induced spontaneous gating in \( \alpha 1 \)-GlyRs (32), may also have exerted a direct or allosteric effect on this site.

The possible agonist-binding roles of the GlyR \( \alpha 1 \)-subunit complementary (D, E, and F) domains have not yet been investigated. This constitutes a significant gap in our understanding of glycine binding mechanisms, as it is currently unclear whether bound glycine molecules are coordinated by adjacent subunits.

The possible agonist-binding role of the \( \beta \)-subunit has also received scant attention. A study on \( X e n o p u s \) oocyte-expressed GlyRs found that the incorporation of \( \beta \)-subunits reduced the Hill coefficient for glycine activation from 4.2 in \( \alpha 1 \)-homomers to 2.4 in \( \alpha 1 \beta \)-heteromers (46). This implied that \( \beta \)-subunits did not contain glycine binding sites. However, not all studies have observed a change in Hill coefficient upon \( \beta \)-subunit incorporation (e.g., Ref. 154). Interestingly, a single-channel study investigating the effect of the \( \beta \)-subunit startle disease mutation G229D found that it disrupted the agonist binding affinity (314). Because G229 lies in loop C (Fig. 2), this effect was most likely mediated by either a direct or allosteric disruption of a \( \beta \)-subunit glycine binding site. Clearly, further experiments are required to clarify the agonist-binding role of the \( \beta \)-subunit.

3. Physical basis of agonist binding

There is abundant evidence that the ACh-nAChR binding reaction is mediated mainly by noncovalent "cation-π" electrostatic interactions (430). In this system, the aromatic side chains of phenylalanine, tyrosine, or tryptophan contribute a negatively charged π surface while
the cation is provided by the agonist. In the α1-nAChR subunit, the ACh quaternary nitrogen interacts directly with Y149 by this mechanism (434). The corresponding GlyR α1-subunit residue Y161 is conserved in all GlyR subunits. By analogy with the nAChR, Y161 could conceivably form a cation π interaction with the glycine amine nitrogen.

Glycinergic agonists have been subjected to a number of structure-activity investigations, with the most notable being that of Schmieden and Betz (336). This study tested the agonist and antagonist potencies of a range of α- and β-amino acids. Agonist activity was found exclusively to be a property of those molecules where the amino and acidic moieties existed in a cis-conformation (Fig. 5). One molecule (nipecotic acid) that was locked into a trans-conformation exhibited only antagonist activity. However, β-amino acids (e.g., taurine) that randomly flicker between both conformations displayed both agonist and antagonist activity (Fig. 5). This model predicts a specific antagonist recognition site in a common ligand binding pocket that is accessible only by trans-isomers (336). By binding in the trans-conformation, β-amino acids may either stabilize the closed state or sterically prevent glycine from binding in the pocket, or both. This model also predicts that the partial agonist activity of taurine results from a fraction of molecules binding as antagonists and another fraction binding as agonists at the same receptor. Although attempts to identify a putative antagonist contact site have proven inconclusive to date (151, 339), this interesting model is certainly worthy of further consideration.

D. Structural Changes Accompanying Activation

1. The ligand-binding domain

Functional data suggest that nAChR activation is mediated by global intersubunit movements (74). A recent structural analysis of the nAChR by Unwin et al. (377) has provided more direct support for such a model. This group interpreted low-resolution electron diffraction images of Torpedo nAChRs obtained in both the closed and open states using the AChBP crystal structure as a template. In modeling the structural changes, they divided the α-subunit into inner and outer parts and considered each separately. The inner part (which includes all residues up to the conserved cysteine loop) is so-called because it faces the channel vestibule and contains most of the intersubunit contact points plus agonist-binding domain A. The outer part (which comprises β-sheets 7–10) includes the agonist-binding domains B and C. Upon agonist binding, the outer part was found to undergo an upwards tilt around an axis parallel with the membrane plane. Simultaneously, the inner part rotated ~15° in a clockwise direction (when viewed from the synapse) around an axis perpendicular to the membrane plane. Being essentially a combination of rigid body movements, the largest residue displacements (up to 2 Å) occurred at the subunit interfaces. The rotation of the inner sheets is viewed as the crucial event in transmitting agonist-binding information to the channel gate (377). The inner sheets contain two loops, the conserved cysteine loop and loop linking the first and second β-sheets (referred to hereafter as loop 2), that are ideally located to interact directly with the TM2-TM3 domain (52, 267). Interactions between these loops and the TM2-TM3 domain appear crucial to the GlyR activation process, and the extensive body of evidence implicating these regions in receptor gating will now be considered.

2. The TM2-TM3 domain

The structural changes initiated in the ligand-binding domain are transmitted to the membrane-spanning domains, where they culminate in a change in conformation of TM2. As stated above, one such contact point is the “TM2-TM3 domain” which extends from R271 to D284 in the GlyR α1-subunit. Although originally thought to comprise an extended extramembranous loop, the Miyazawa TM structure now reveals this domain to comprise the extramembranous portions of the TM2 and TM3 α-helices plus a short connecting loop (267).

A signal transduction role for single residues in this domain was first suggested by studies on the GABA<sub>C</sub>R (204), the GlyR (305), and the nAChR (56). A systematic study subsequently proposed a structural role for the entire α1-GlyR TM2-TM3 domain in the gating process (237). This conclusion was based on the observation that
several naturally occurring human disease mutations, plus several more alanine-substitution mutations, scattered throughout this domain acted similarly in reducing the agonist efficacies of taurine and β-alanine relative to glycine. Because β-alanine and taurine retained high potency as antagonists, their agonist binding affinities were considered to be little affected. Thus, because a number of mutations throughout this domain predominantly impaired E, the whole domain was hypothesized to comprise a structural element of the receptor activation pathway (237). A subsequent single-channel study on one of the tested mutants (K276E) supported this interpretation (228). A more direct test of this theory required an investigation into whether the region moves during activation. Accordingly, SCAM was employed to probe for changes in its surface accessibility between the closed and open states. The results did reveal an increased surface accessibility of the NH2-terminal (α-helical) half of the domain in the open state (234), consistent with a conformational change during gating. Several approaches have since been used to implicate various TM2-TM3 residues in the gating of other LGICs including the nAChR (75, 141, 142, 320, 321) and GABA\(_\text{A}\)R (37, 44, 113, 181, 285).

Recent studies have begun to shed light on the specific structural role of this domain. The Auerbach group examined the linear free-energy relationships (LFERs) of nAChRs incorporating mutations in various positions (142). They showed that the energy transition experienced by a TM2-TM3 residue was intermediate between those experienced by residues at the binding site and the activation gate. This was interpreted to mean that the TM2-TM3 domain is positioned midway along the agonist-induced “conformational wave” that proceeds from the binding site to the activation gate. Another study, undertaken on the GABA\(_\text{A}\)R, employed a mutant cycle analysis approach to identify an electrostatic attraction between the negatively charged residue D149 and the positively charged residue K279 (181). D149 lies in the conserved cysteine loop, whereas K279 lies in the TM2-TM3 domain. A cysteine cross-linking approach further supported the idea of a closer association between these residues in the open state (181). Together, the results suggested that channel activation is accompanied by an increased electrostatic attraction between these two residues. However, the electrostatic attraction between the corresponding charged residues (D148 and K276) in the α1-GlyR is weaker, suggesting other interactions may also contribute to channel activation (1, 340).

Structural and functional analyses have also revealed a close interaction between the TM2-TM3 domain and loop 2 of the ligand-binding domain (181, 267). Indeed, the Miyazawa TM structure suggests that one loop two hydrophobic side chain fits into the end of the nAChR TM2 helix like “a pin in a socket.” Charged loop 2 residues have also been implicated in gating the α1-GlyR (1, 340) and the GABA\(_\text{A}\)R (181). Clearly, substantial gaps remain in our understanding of how agonist signals are transduced from the binding site to the activation gate of the GlyR. Although it is possible that different LGIC members employ different coupling mechanisms, a common coupling mechanism for ρ1-GABACRs and α1-GlyRs is suggested by the chimeric studies of Mihic and colleagues (262).

3. The TM1-TM2 domain

Systematic mutagenesis of residues in the intracellular TM1-TM2 domain suggested this domain may also be involved in α1-GlyR gating (237). As discussed in section 3D, several mutations in this region also affect desensitization. These observations imply that GlyR gating is very sensitive to structural perturbations in this domain. It seems likely, therefore, that this region might also move during GlyR activation. Further experiments are required to test this hypothesis.

4. The membrane-spanning domains

Investigations into the structural rearrangements of TM2 have revealed two major features that will be considered in turn. The first feature originally stemmed from the observation that the TM2 domain of the Torpedo nAChR incorporated a centrally located kink that appeared to form the channel gate (376). Concurrent SCAM studies (7) also provided evidence for a discontinuity in the α-helix around the central (L9) position. Such a discontinuity is likely to introduce a degree of conformational flexibility because some of the H-bonds responsible for maintaining the α-helical structure may be broken. This discontinuity may therefore serve as a swivel joint to permit the outer half of TM2 to move asynchronously with the inner half. In other words, gating may involve a backbone rearrangement in the vicinity of L9'. This has indeed been suggested by SCAM studies (7), LFER analysis (83), molecular dynamics simulations (216), and a study that introduced unnatural, backbone-altering mutations into TM2 (104).

The second major feature of TM2 gating also concerns the 9’ position. The muscle nAChR L9'T mutation dramatically affects the desensitization rate, the size of spontaneous leakage currents, and the effects of allosteric modulators (reviewed in Ref. 74), implying complex (possibly global) effects on channel gating mechanisms. Furthermore, mutating the 9’ leucines to small polar residues (serines or threonines) had an equal effect on the ACh dose-response regardless of which subunit was mutated (110, 206). As binding sites exist at only two of the five subunit interfaces, these results implied that neighboring nAChR subunits interact allosterically via their respective L9’ residues. Recent evidence suggests that GlyR α- and β-subunits interact in a similar manner (344). The Miyazawa TM domain structure (267) provides some
insight into how these interactions might occur. The structure suggests the existence of hydrophobic bonds between the 9’ and 10’ residues of adjacent subunits. These bonds appear to "balance" the L9’ residues into a fivefold radially symmetrical arrangement that holds the channel closed. It is likely that agonist-induced conformational changes asymmetrically disrupt some of these bonds, leading to a collapse of symmetry and a simultaneous conversion of all TM2 domains to the activated state. Mutations to one or more L9’ residues may achieve a similar effect.

In summary, the main features of agonist-induced TM2 movements are that 1) its midpoint acts as a swivel, and 2) this swivel point mediates interactions between adjacent subunits. Agonist-induced backbone rearrangements at this position may thereby lead to a concerted conformational change at either a centrally located or an intracellularly located gate (376, 404).

Functional evidence suggests that TMs 1, 3, and 4 may also be involved in LGIC gating. Single-channel kinetic analysis of mutations incorporated into each of these domains suggests that some residues may have specific roles in gating the nAChR (47, 89, 104, 388). SCAM analysis has also provided evidence for state-dependent structural rearrangements of TM1 and TM3 in the nAChR and GABA_αR, respectively (6, 400–402, 432). However, insufficient information is available to date on any LGIC member to form a coherent picture of how these domains contribute to activation. The recently elucidated nAChR TM domain structure (267) now permits the design of more specific experiments to investigate these mechanisms.

5. The β-subunit

The role of the β-subunit in α1β-GlyR gating was recently investigated by incorporating mutations into corresponding positions in α1- and β-subunits and comparing their effects on receptor function (344). Although cysteine-substitution mutations to residues in the NH₂-terminal half of the α1-subunit TM2-TM3 loop dramatically impaired gating efficacy (234), the same mutations exerted little effect when incorporated into corresponding positions of the β-subunit (344). Furthermore, although α1-subunit TM2-TM3 loop cysteines were modified by cysteine-specific reagents (234), the corresponding β-subunit cysteines showed no evidence of reactivity (344). These observations suggest structural or functional differences between α1- and β-subunits. However, the incorporation of the L9’T mutation into the β-subunit dramatically increased the glycine sensitivity (344), suggesting an allosteric modulatory effect on the α1-subunit. Thus β-subunit conformational changes do contribute to the activation of the GlyR, although their involvement in this process is significantly different to that of the α1-subunit.

VI. GLYCINE RECEPTOR MODULATION

A. Phosphorylation

Phosphorylation can cause long-term changes in the functional properties of ion channels, and an abundance of evidence implicates such mechanisms in various forms of synaptic plasticity (177). Intracellular signaling pathways determine the phosphorylation state of proteins by coordinating the activities of protein kinases, which induce phosphorylation, and phosphatases, which reverse it. All of the functional phosphorylation sites on LGICs have been mapped to the major intracellular loop (360). As discussed above, LGIC subunits exhibit a low degree of sequence homology in this region, and this underlies the subunit-specific distribution of phosphorylation consensus sites (Fig. 2). It has recently become apparent that clustering and cytoskeletal anchoring proteins can influence the proximity of kinases and phosphatases to the LGIC intracellular domains (226, 360). Thus the ability of an LGIC to be phosphorylated depends not only on its subunit composition but also its proximity to the appropriate enzymes. In addition, kinases and phosphatases may have indirect effects on the GlyR by controlling the phosphorylation state of modulatory proteins. This diversity could lead to tissue-specific differences in the propensity of a given GlyR isoform to be phosphorylated.

1. Protein kinase A

Contrasting effects of cAMP-dependent phosphorylation on GlyR current magnitudes have been reported in neurons from various parts of the brain (219). Although the differences may be related to the phosphorylation of GlyR modulatory proteins (or possibly to tissue-specific or nontissue-specific effects of pharmacological probes), biochemical experiments strongly suggest that spinal cord GlyR α-subunits themselves are directly phosphorylated in vitro (378). However, most GlyR α-subunit isoforms do not contain protein kinase A (PKA) phosphorylation consensus sequences. The exception to this is the α1ins splice variant which contains an eight-amino acid insert (. . . SPMLNFLQ. . . ) in the large intracellular domain, and the first residue of this insert may serve as a phosphorylation site (244). As shown in Figure 2, the β-subunit also contains a PKA consensus site at a different position (T363) in the TM3-TM4 domain (137). However, it is yet to be determined whether the α1ins or the β-subunit sites are directly phosphorylated and, if so, whether phosphorylation induces changes in receptor function. As noted by Legendre (219), it is possible that the contradictory effects of cAMP-dependent phosphorylation may be explained by the differential expression of α1ins- and β-subunits throughout the central nervous system (245). Whether this is true, and whether cAMP-dependent phos-
phorylation has a physiological role at glycinergic synapses, are important questions for future research.

2. Protein kinase C

Physiologically, protein kinase C (PKC) is activated by increases in intracellular calcium or diacylglycerol. The GlyR α1-subunit contains a PKC phosphorylation consensus sequence at S391 in the TM4 domain (323). Consistent with this, the spinal cord GlyR α-subunit was shown to be phosphorylated by PKC in an in vitro assay (378). Support for a functional role for S391 is provided by the observation that the homologous residue in the GABAAR β-subunit is phosphorylated by PKA and PKC (274). Surprisingly, however, the corresponding residue in the Torpedo nAChR appears inaccessible to the protein surface (267). The GlyR β-subunit also contains a putative PKC phosphorylation consensus site at position 389 in the TM3-TM4 domain (137), although its functional status is yet to be confirmed. Pharmacological manipulations aimed at stimulating or inhibiting PKC have revealed contrasting effects on glycine-activated currents in a variety of neuron types (219). As discussed above, these differential effects may be due to a multitude of causes, including, in some cases, nonspecific effects of pharmacological agents (e.g., Ref. 281). Our current understanding of PKC-dependent phosphorylation is further complicated by the fact that differences have also been observed in supposedly identical recombinant GlyRs. For example, PKC was found to potentiate glycine currents in Xenopus oocyte-expressed α1- and α2-homeric GlyRs (281). In apparent contrast, PKC activators did not affect current magnitude in α1-GlyRs expressed in either Xenopus oocytes (253) or HEK293 cells (128). However, the later study reported that PKC activation accelerated the onset of and slowed the recovery from desensitization (128). A definitive understanding of PKC-dependent phosphorylation processes in the GlyR will require functional assays involving site-directed mutagenesis of putative phosphorylation sites and biochemical assays to directly determine the receptor phosphorylation state.

Interestingly, PKC activation has been shown to increase the potentiating effects of ethanol in recombinant α1-GlyRs (253). Ethanol was shown to potentiate, inhibit, or have no effect on glycine-activated responses in 35, 45, and 20%, respectively, of a large sample of ventral tegmental area (VTA) neurons (422, 423). In the population of VTA neurons where ethanol induced inhibition, PKC activators seem to compete with ethanol for a common inhibitory site (364). A pharmacological study on those VTA neurons where ethanol induced potentiation, activation of the PKC epsilon isoform was found to increase potentiation magnitude (173). Together, these findings raise the possibility of a specific allosteric linkage between the phosphorylation site and the alcohol binding site.

3. Protein tyrosine kinase

Lavendustin A, a protein tyrosine kinase (PTK) inhibitor, reduced glycine currents in hippocampal and spinal neurons, whereas intracellular application of c-Src, an endogenous tyrosine kinase, increased glycine current magnitudes (57). The current increases, which were mediated by a leftward shift in the glycine EC_{50}, were accompanied by an enhanced desensitization rate (57). Similarly, effects of c-Src were observed on recombinant α1β-GlyRs expressed in HEK293 cells, but not in recombinant α1-GlyRs expressed in the same cells. Mutation of a putative tyrosine phosphorylation site (Y413F) in the large intracellular loop of the β-subunit (137) abolished the effects of several tyrosine phosphorylation modulators, suggesting this site is functionally phosphorylated (57).

Although these experiments provide strong circumstantial evidence for β-subunit phosphorylation, biochemical confirmation is required to eliminate the possibility of allosteric effects induced by the Y413F mutation.

B. Modulators of Possible Physiological Relevance

1. Zinc

A) A PUTATIVE PHYSIOLOGICAL ROLE. Zinc is concentrated into round clear presynaptic vesicles in the central nervous system and is released into the synaptic cleft by nerve terminal stimulation (20, 120, 164). During synaptic stimulation, zinc is thought to reach a peak external concentration of >100 μM (20, 120, 385). At such concentrations, zinc is able to modulate a wide variety of pre- and postsynaptic ion channels (349). Low (0.01–10 μM) concentrations of zinc potentiate glycine currents by increasing the apparent glycine affinity without changing the saturating current magnitude, whereas higher concentrations (>10 μM) inhibit the current by reducing the apparent glycine affinity (213). This pattern of zinc action is seen in native receptors (42, 63, 152, 359, 365) as well as in recombinant α1-, α2-, and α1β-GlyRs (213). In addition to increasing the magnitude of glycnergic IPSCs, low concentrations of zinc have also been shown to prolong their duration and frequency (211, 359), with both effects presumably being due to the increased glycine sensitivity.

The role of zinc has been most thoroughly investigated at the mossy fiber glutaminergic synapse in the hippocampus (120). However, some suggestions have recently emerged that zinc may also have a physiological role at glycnergic synapses. First, an ultrastructural study has found evidence for zinc and glycine colocalization in individual presynaptic terminals in the spinal cord (39). Second, at glycnergic synapses of the intact zebrafish...
hindbrain, zinc chelators decreased the amplitude, duration, and frequency of glycinergic IPSCs, whereas zinc application had the opposite effect (359). However, it remains to be established whether zinc is coreleased with glycine at concentrations high enough to modulate GlyR function.

It is important to note that even strongly inhibiting zinc concentrations (≥30 μM) causes an initial transient potentiation followed by the slowly developing inhibition (235). The duration of this transient potentiation, which is of the order of 1 s (203, 235), easily exceeds that of a typical glycinergic IPSC. However, zinc inhibition stabilizes much more rapidly in the absence of agonist (235) so that if an inhibiting concentration of zinc reaches the GlyR before glycine does, then only inhibition is seen (211, 235). Together, these observations mean that high zinc concentrations may have opposite effects on glycinergic IPSC magnitude depending on whether the zinc reaches the receptor before or after glycine. This should be considered in models of zinc action on glycinergic synapses.

No other metal ion has convincingly been shown to mimic the biphasic action of zinc. However, the potentiating site is recognized by several metal ions with the potency sequence: zinc > lanthanide > lead > cobalt (96, 203), whereas the inhibitory site exhibits the potency sequence: zinc > copper > nickel (96, 203).

B) MOLECULAR MECHANISM OF ACTION. In most proteins, zinc ions are coordinated by nitrogen, sulfur, or oxygen atoms found in the side chains of histidine, cysteine, aspartic acid, and glutamic acid residues (131). Zinc binding sites are usually comprised of two to four residues, and the affinity of a site depends on the number of residues, their relative positions, and the local electrostatic environment (21, 317). Several lines of evidence suggest that the GlyR zinc potentiating and inhibitory binding sites are physically discrete.

C) INHIBITION. In contrast to the rapid onset of potentiation, zinc inhibition is slow to develop (235). However, as noted above, this inhibition develops much more rapidly in the absence of agonist (235). This result suggests that glycine-induced activation is accompanied by a structural change in this location and that zinc acts by stabilizing the closed conformation. A single-channel study found high (50 μM) zinc concentrations reduced α1-GlyR open probability by reducing mean channel open time and the relative abundance of long channel bursts (212). It concluded that zinc increases the rate at which the channel exits from the open state, supporting the view that zinc stabilizes the closed state.

Zinc inhibition of the recombinant α1-GlyR was found to be selectively abolished by either reducing the pH or by pretreatment with diethylpyrocarbonate, a histidine-specific modifying agent (158). Because both treatments effectively reduce the ability of zinc to bind with histidine imidazole rings, histidines were implicated in the complexation of zinc at its inhibitory site. Mutations to either H107 or H109 were subsequently shown to abolish zinc inhibition, strengthening the case that these residues formed an inhibitory binding site (158). Histidine α-carbonyl oxygen atoms need to be within 13 Å of each other to permit their imidazole rings to coordinate a zinc ion (21). Because the histidines are separated by only one residue, it is certainly feasible that the zinc ions could be coordinated within individual α-subunits. However, the α-carbonyl oxygens of the homologous residues in adjacent AChBP subunits are separated by 7.7 Å (52). With the assumption that this structure is reasonably well conserved in the GlyR, it is plausible that zinc ions could be coordinated by adjacent α-subunits. This possibility was tested by coexpressing α1-subunits containing the H107A mutation with α1-subunits containing the H109A mutation (276). Although sensitivity to zinc inhibition is markedly reduced when either mutation is individually incorporated into all five subunits, the GlyRs formed by the coexpression of H107A mutant subunits with H109A mutant subunits exhibited an inhibitory zinc sensitivity similar to that of the wild-type α1-homomeric GlyR. This constitutes strong evidence that inhibitory zinc is coordinated at the interface between adjacent α-subunits, but does not rule out the possibility that zinc may also be coordinated within α1-subunits. No evidence was found for β-subunit involvement in the coordination of inhibitory zinc, indicating that a maximum of two zinc-binding sites per heteromeric receptor is sufficient for maximal zinc inhibition (276). This region of the α1-subunit aligns poorly with AChBP due to the existence of three additional α1-subunit residues. Homology models were constructed using several alignments, and only one of these produced a plausible zinc binding site (276). The successful alignment is depicted in Figure 2, and the modeled structure of this site is shown in Figure 3B.

D) POTENTIATION. Low (5 μM) zinc concentrations increase the open probability of the α1-GlyR by increasing both the opening frequency and the mean burst duration (212). The authors concluded that the channel opening and closing rates were not significantly affected and that zinc acted primarily by slowing the rate of glycine dissociation from the binding site. This suggests an allosteric effect of zinc that improves the fit of glycine to its site. In contrast, zinc had a dual effect on taurine-gated currents. It not only slowed the rate of taurine dissociation from its binding site, but increased the rate at which bound taurine could activate the channel (212). Another group showed that mutations to various residues in the TM1-TM2 and TM2-TM3 domains abolished zinc potentiation of glycine currents, while leaving zinc potentiation of taurine currents intact (235). These results suggest that the allosteric linkage between the zinc potentiating site and the glycine binding site or transduction pathway was
selectively disrupted. The results can be reconciled with those of Laube et al. (212) by proposing that the mutations selectively disrupted the ability of zinc to enhance the glycine affinity.

Analysis of chimeric constructs of α1- and β-subunits implicated D80 as a specific determinant of zinc potentiation (213). Indeed, mutations (D80A, D80G) to this residue disrupted zinc potentiation of glycine currents (212, 235), whereas mutations to neighboring aspartic acid residues (D81A, D84A) had no effect (212). However, because D80A did not abolish zinc potentiation of taurine-gated currents (235), its putative role as a zinc binding site must be queried. The potentiating effect of zinc was also abolished by the H109A mutation (158) as well as several mutations in the TM1-TM2 and TM2-TM3 linker domains (235). Unfortunately, this widespread distribution of site-directed mutations that abolish zinc potentiation does not bode well for the use of this approach in identifying the GlyR zinc potentiating site. The lack of zinc voltage dependence and rapid reversibility of the potentiating effect (212, 235) indicate that the potentiating binding site resides in an extracellular domain.

2. Calcium

Calcium current influx through glutamate-activated channels causes a rapid (<100 ms) and transient elevation of glycine current magnitude (124, 411, 412) that may have an important physiological role in modulating the gain of glycineric transmission. Based on a pharmacological analysis on GlyRs expressed on rat spinal sensory neurons, Xu and colleagues (411, 412) proposed that the effect was mediated by calcium activation of calmodulin-dependent protein kinase II and calcineurin. However, Fucile et al. (124), who examined a similar effect in both cultured spinal neurons and recombinant α1-GlyRs, found it was resistant to a variety of manipulations designed to disrupt phosphorylation, dephosphorylation, and G protein-dependent processes. These differences may relate to the different origins of the neurons studied and could be indicative of multiple mechanisms contributing to this effect. Another recent study, conducted on VTA neurons, found the calcium-dependent potentiation to be antagonized by ethanol (438).

In the effect seen by Fucile et al. (124), single-channel analysis suggested the calcium-dependent factor exerted complex effects on both the glycine binding affinity and the gating rate (124). Because the calcium-induced increase did not reverse in inside-out patches, it was considered likely to be mediated by the calcium-induced removal of a soluble cytoplasmic intermediate from the receptor internal surface. Although the identity of this intermediate remains elusive, it appears to be endogenously expressed in HEK293 cells as well as in spinal neurons (124).

3. pH

Transient increases in extracellular pH occur in response to the activation of anionic LGIC receptors (65). The mechanism is most likely related to the high HCO$_3^-$ permeability of anionic LGICs. When the pore opens it is likely that HCO$_3^-$ exit the cell, causing intracellular acidification and extracellular alkalization (65). In recombinant α1- and α1β-GlyRs, the glycine EC$_{50}$ is significantly increased as the pH is lowered from 7.5 to 6.0 (64). This effect appears to be mediated by a specific interaction with the GlyR extracellular domain as it is abolished by the α1-subunit mutations H109A, T112A, and T112F, but is not affected by other mutations to T112 or by mutations to neighboring negatively charged residues (64). Mutation to the β-subunit residue that corresponds to T112 (i.e., T135A) was also found to reduce proton sensitivity (64). Thus pH sensitivity appears to be specific to the receptor region that houses the zinc inhibitory binding site. However, as threonines are not ionizable, it is unlikely that they directly form the proton acceptor site.

4. Neurosteroids

Neurosteroids are hormones that are synthesized in central nervous system glia and neurons from cholesterol or blood-borne steroidal precursors (24, 357, 372). Although neuroactive steroids produced in the peripheral steroidogenic glands can easily access the brain, it is thought that steroids produced in the central nervous system have important paracrine roles (207). Although the complex behavioral effects of neurosteroids have been attributed primarily to GABA$_A$ and NMDA receptors (24, 103, 207, 357), potent neurosteroid actions have been observed on native and recombinant GlyRs. Antagonistic effects of progesterone, its precursor pregnenolone (PREG), and pregnenolone sulfate (PREGS) were first shown on glycine currents in cultured spinal neurons (407, 408). Another early report showed that glycine sensitivity in the rat optic nerve was increased by several corticosteroids at concentrations of 1–10 μM (299). On the other hand, both α1- and α1β-GlyRs were found to be insensitive to the action of 5α-pregnen-3α-ol-20-one (295). Using a more systematic approach, Maksay et al. (243) examined a battery of neurosteroids on recombinant α1-, α2-, α4-, α1β-, and α2β-GlyRs. Both PREGS and dehydroepiandrosterone sulfate (DHEAS) inhibited all of these receptors with inhibitory constant (Ki) values of 2–20 μM, with the compounds showing only a modest degree of subunit specificity. Of particular note, DHEAS inhibited the α2-GlyR with an IC$_{50}$ of 4.2 μM, whereas its IC$_{50}$ at the α1β-GlyR was 21.2 μM. PREG caused a ~25% increase in the α1-GlyR current with an EC$_{50}$ of 1.4 μM, but had no effect on α1β- or α2-GlyRs (243). On the other hand, progesterone inhibited the α2-GlyR current by 23% with an IC$_{50}$ of 20 μM, while exerting no effect on the α1- and
Aβ-GlyRs (243). Given the $\alpha 2 \rightarrow \alpha 1 \beta$ subunit switch that occurs after birth in the rat, the differential effects of DHEAS and progesterone on the $\alpha 2$- and $\alpha 1 \beta$-GlyRs may have physiological relevance for neuronal development.

Although the molecular determinants of neurosteroid action at the GlyR have not yet been determined, some progress has been made on the GABA$_{\text{A}}$R$_{\beta}$ subunit. On the basis of a chimeric study on alphalaxone-sensitive and -insensitive GABA$_{\text{A}}$R subunits, a necessary determinant of neuropeptide receptor action was isolated to the NH$_2$-terminal half of TM2 (318). Consistent with this, the V2'S mutation in the GABA$_{\text{A}}$R $\alpha 1$-subunit reduced the potency of PREGS block by 30-fold (11). Particularly since the GlyR $\gamma 2$' residue is known to affect the potencies of other GlyR modulators (see below), it is a promising candidate as a specific determinant of neurosteroid action in the GlyR.

Finally, it has long been known that that the synthetic steroid RU 5135 displaces $[^3\text{H}]$strychnine binding with a remarkably high (5 nM) affinity (48). Because the mechanism of action of this ligand has never been investigated, it is not known whether it shares a similar mode of action to the endogenous neurosteroids.

### 5. G protein βγ-subunits

The irreversible activation of G proteins by nonhydrolyzable GTP analogs has recently been shown to exert potent effects on the GlyR. These reagents cause both a leftward shift in the glycine EC$_{50}$ of recombinant $\alpha 1$-GlyRs and a prolongation of glycineergic synaptic currents in cultured spinal neurons (425). These effects are most likely mediated by a direct interaction of a G protein βγ-subunits with the GlyR $\alpha 1$-subunit because 1) βγ-subunits coimmunoprecipitate with the GlyR $\alpha 1$-subunit, and 2) addition of βγ-subunits to the intracellular membrane surface reversibly increases GlyR channel activity (425). These results suggest that G protein-coupled receptor activation may be important in vivo for regulating the gain of glycineergic synaptic transmission.

### C. Molecular Pharmacology

#### 1. Strychnine and analogs

The plant alkaloid strychnine (Fig. 6) is a highly selective and extremely potent competitive antagonist of glycine, β-alanine, and taurine with a dissociation constant of 5–10 nM (81, 427, 428). Strychnine sensitivity is currently the most definitive means of discriminating glycineergic from GABAergic synaptic currents. Because of its high affinity and specificity, $[^3\text{H}]$strychnine has been widely used in radioligand displacement studies to investigate the potencies and allosteric actions of other GlyR ligands (241). Strychnine has been subjected to several structure-activity investigations (162, 239, 249). However, no analog has ever been shown to possess a higher apparent affinity than strychnine. Similarly, no strychnine-like ligands have yet been shown to exert agonist or allosterically enhancing properties. Surprisingly, however, strychnine has been shown to behave as an agonist in modified α7-homomeric nAChRs (286).

Early evidence from GlyR protein modification experiments indicated that the strychnine and glycine binding sites were mutually interactive but not identical (249). This interpretation has also been reached as a result of site-directed mutagenesis experiments. The first of these experiments stemmed from the observation that the $\alpha 2^\alpha$-splice variant subunit had a strychnine IC$_{50}$ that was ~500 times higher than those of the $\alpha 2$- or $\alpha 1$-subunits (202). Strychnine sensitivity was restored by incorporating the E167G mutation into the $\alpha 2^\alpha$-subunit, thus reversing the only nonconserved amino acid between the $\alpha 2^\alpha$- and $\alpha 2$-subunits (202). This residue aligns with G160 in the GlyR $\alpha 1$-subunit. As expected, strychnine insensitivity was conferred to the $\alpha 1$-subunit by the reverse G160E mutation (381). Mutation to the adjacent $\alpha 1$-subunit residue Y161A also abolished strychnine sensitivity (381), although the more conservative mutation Y161F did not (338). Photoaffinity labeling experiments localized another crucial strychnine binding element to either Y197 or Y202 of the $\alpha$-subunit (322). Subsequently, Vandenberg and colleagues (309, 381, 382) identified K200 and Y202 as strychnine binding sites on the basis of functional analysis of $\alpha 1$-GlyRs incorporating site-directed mutations at these positions. Because glycine also binds to common or adjacent residues in both of these strychnine-binding domains (see above), it is reasonable to conclude that strychnine and glycine share overlapping but nonidentical binding sites in principal ligand binding domains B and C. This, of course, provides a structural basis for the competitive antagonist behavior of strychnine (382).

Substances that act purely as competitive antagonists are comparatively rare. Generally, it would be expected that any substance that binds to a receptor would also bias the conformational equilibrium towards a particular state. However, the possible allosteric effects of strychnine have yet to be considered. One way of doing so would be to determine the effects of low strychnine concentrations on modified GlyRs that spontaneously gate in the absence of agonist.

#### 2. Picrotoxin and analogs

Derived from plants of the moonseed family, the convulsant alkaloid picrotoxin is also widely used to discriminate GABAergic from glycineergic currents (348). Picrotoxin (PTX) strongly inhibits GABA$_{\text{A}}$Rs at 1–10 μM concentrations, whereas GlyRs in vivo are considerably less sensitive. PTX comprises an equimolar mixture of picrotoxinin and picrotin. As shown in Figure 6, these
compounds differ only in the structure of the terminal isoprenyl group, which in the case of picrotin is hydrated to remove the double bond. Picrotoxinin potently inhibits the GABA<sub>A</sub>R, whereas picrotin is generally inactive at this receptor. This behavior correlates well with the relative systemic toxicities of the two substances. PTX inhibition of the GABA<sub>A</sub>R is use dependent (i.e., inhibition reaches steady-state at a faster rate in the open state) and non-competitive, and its inhibitory potency is highly sensitive to TM2 mutations (see below). This combination of properties has frequently led to PTX being classified as a channel blocker. However, several lines of evidence (e.g., Refs. 277, 296) have firmly established PTX as an allosteric inhibitor of the GABA<sub>A</sub>R.

In 1992, Pribilla et al. (298) reported that <i>/H9251/H9252</i>-hetero-meric GlyRs were much less sensitive to PTX inhibition than were <i>/H9251</i>-homomeric GlyRs. This result was independent of which <i>/H9251</i>-subunit (<i>/H9251</i><sub>1</sub>, <i>/H9251</i><sub>2</sub>, or <i>/H9251</i><sub>3</sub>) was investigated. They also showed that inserting the <i>/H9251</i><sub>1</sub>-subunit TM2 into the <i>/H9252</i>-subunit bestowed high PTX sensitivity to <i>/H9251</i><sub>1</sub>/H9252-GlyRs. These findings were important for two reasons. First, by establishing TM2 as a crucial determinant of PTX sensitivity, they prompted an intensive investigation into the molecular basis of PTX action in various LGIC members (106, 109, 150, 389, 410, 433). Second, they established PTX sensitivity as a standard pharmacological tool for identifying the presence of β-subunits in recombinant and native GlyRs. Although several other compounds also display subunit sensitivity (see below), there is as yet no better tool than PTX for this purpose.

It was subsequently shown that picrotin and picrotoxinin were equally efficacious in inhibiting <i>/H9251</i>-GlyRs (236). The same study demonstrated that PTX inhibition was not use dependent and that its inhibition was “competitive,” meaning that its potency decreased as agonist concentration increased. Both behaviors are uncharacteristic of channel blockers. An allosteric mode of action was confirmed by the findings that the R19'L and R19'Q startle disease mutations transformed PTX into an allosteric potentiator at low concentrations (<3 μM) and a
noncompetitive, slow-onset inhibitor at higher concentrations (236).

A series of studies on the GABA$_A$R, GABA$_C$R, and GluClR established the 2‘ and 6‘ residues as crucial determinants of PTX sensitivity (106, 109, 150, 389, 410, 433). A common feature in all of these studies was that a ring of 6‘ threonines was invariably required for high PTX sensitivity. Although all GlyR $\alpha$-subunits contain a threonine at this position, the $\beta$-subunit contains a phenylalanine. As anticipated, a range of mutations to T6‘ in the GlyR $\alpha$1-subunit, including the $\alpha \rightarrow \beta$ substitution T6‘F, greatly diminished the inhibitory potency of PTX and related compounds (341, 343, 356). In addition, incorporating a threonine into the $\beta$-subunit 6‘ position restored high PTX sensitivity to the $\alpha1\beta$-GlyR (341), although a range of other $\beta$-subunit 6‘ mutations had no such effect (343). Does this highly specific requirement mean that T6‘ is the PTX binding site? Despite a molecular modeling study finding that PTX can fit into this part of the pore and that T6‘ hydrogen bonds could plausibly coordinate a PTX molecule (435), it is premature to draw this conclusion.

The PTX-competitive compound $\alpha$-ethyl-$\alpha$-methyl-$\gamma$-thiobutyrolactone ($\alpha$EMTBL; Fig. 6) was found to potentiate glycine responses in $\alpha$1-GlyRs but inhibit them in $\alpha$3-GlyRs (356). The TM2 domains of these subunits are conserved with the exception of the 2‘ residue: in the $\alpha$1-subunit it is a glycine, but in the $\alpha$3-subunit it is an alanine. The inhibition seen in the $\alpha$3-GlyR is abolished by the T6‘F mutation, although the potentiation seen in the $\alpha$1-GlyR is not affected by this mutation (356). Moreover, incorporating the $\alpha1$ 2‘ residue into the $\alpha3$-subunit (via the A2‘G mutation) converts $\alpha$EMTBL inhibition into potentiation. To interpret these results in terms of binding sites, one would have to postulate a site that toggles between inhibitory and potentiating depending on the identity of residues at the 2‘ and 6‘ positions. The existence of two discrete sites (an inhibitory site in the pore and a potentiating site elsewhere) seems equally plausible. This would require that a mutation that abolishes the PTX or $\alpha$EMTBL inhibitory sites should simultaneously render functional a distant potentiating site. Thus, although they provide little support for a PTX or $\alpha$EMTBL binding site at T6‘, the above results argue strongly for a close allosteric coupling between the 2‘ and 6‘ residues.

The 15‘ residue has also been implicated into this scheme. When the GlyR $\alpha$1-subunit S15‘ was mutated to a glutamine or asparagine, inhibition became use dependent and noncompetitive (92). This is similar to the effect previously seen with R19‘ mutations (236). Because S15‘ and R19‘ mutations abolished the rapid-onset PTX effect that is also abolished by T6‘ mutations, it suggests an allosteric interaction between R19‘, S15‘, and T6‘. Does this imply that S15‘ is the PTX-binding site? After all, evidence summarized below strongly implicates S15‘ as an alcohol and volatile anesthetic binding site.

The current picture regarding the effects of PTX is complex because mutations to residues at the 2‘, 6‘, 15‘, and 19‘ positions can each affect the mode or potency of PTX action. Since all of these mutations have been shown to exert allosteric effects on PTX binding or effector sites, it is uncertain which, if any, is a PTX contact site. It appears that an imaginative approach will be required to convincingly resolve this issue.

Single-channel analysis also reveals complexities in the actions of PTX. In homomeric $\alpha$-GlyRs, it reduced the predominant conductance state from ~80 to 40 pS, with the probability of entering the lower conductance state progressively increasing as the PTX concentration was raised from 1 to 30 $\mu$M (217). In contrast, 30 $\mu$M PTX had no effect on $\alpha\beta$-heteromeric GlyRs, which (perhaps not coincidentally) also exhibited a 40-pS predominant conductance level (217). Higher (100 $\mu$M) PTX concentrations induced flickery kinetics in both $\alpha$- and $\alpha\beta$-GlyRs (217, 426). These observations support the conclusion that PTX acts in an allosteric manner.

In summary, experiments to date have revealed an unusually complex mode of action for PTX. It seems they brought us little closer to formulating testable hypotheses concerning the binding site or mechanism of action of this enigmatic compound.

3. Cyanotriphenylborate

Negatively charged cyanotriphenylborate (CTB; Fig. 6) was chosen as a potential GlyR pore blocker due to its structural similarity with triphenylmethylphosphonium bromide, a classical nAChR pore blocker. CTB was duly shown to act in the predicted manner: its inhibition of the $\alpha$1-GlyR was potent (IC$_{50}$ ~1.3 $\mu$M), use dependent, voltage dependent, and noncompetitive (324). Furthermore, it was not a potent inhibitor of the $\alpha2$-GlyR or $\alpha3$-GlyRs, and replacing the $\alpha1$-subunit 2‘ residue with the $\alpha2$-subunit residue (via the G2‘A mutation) abolished block. Together, these observations provide a strong case for CTB binding in the pore. However, it is not straightforward to conclude that CTB binds to the 2‘ glycine, as it also potently blocked an $\alpha2\beta$-GlyR in which the $\beta$-subunit TM2 domain had been entirely replaced by that of the $\alpha2$-subunit. This paradoxical result demonstrates that CTB sensitivity can reside in a receptor containing only alanines at the 2‘ position, thereby directly refuting the idea that the CTB binding site requires 2‘ glycines. One possibility is that regions apart from the TM2 domain may also contribute to CTB sensitivity (324). Alternatively, $\beta$-subunits may serve the same role as $\alpha$-subunit 2‘ glycines in creating a favorable geometry for the binding of CTB at some level in the pore. Progress in characterizing the CTB...
mechanism of action is currently limited due to its lack of commercial availability.

4. Ginkgolides

Isolated from the leaves, roots, and bark of the Gingko tree, these macrocyclic terpine compounds are widely used in herbal medicine. They also share common structural features with picrotoxinin (170). Ginkgolide B, which is well known as a platelet activating factor antagonist (Fig. 6), is also a specific and potent blocker (IC$_{50}$ ~0.27 μM) of glycine-gated currents in dissociated rat hippocampal pyramidal neurons (192). It is noncompetitive and use dependent, and its inhibitory potency is not affected by the competitive antagonist strychnine. Importantly, when applied externally, its blocking ability increased with cell depolarization, as expected for a negatively charged compound binding in the pore (192). These characteristics establish ginkgolide B as a classical pore blocker. Its molecular determinants of action and subunit specificity have yet to be investigated.

More recently, the effects of several ginkgolides were compared on GlyRs expressed in rat embryonic cortical neuron slices (170). As these GlyRs were insensitive to PTX, they may have comprised predominantly α2β-heteromers. Ginkgolides B, C, and M were found to be more potent than ginkolide A, ginkgolide J, or bilobalide (a related compound from the same tree). In agreement with the earlier study (192), ginkgolide B inhibited the GlyR in a use-dependent, noncompetitive manner and showed specificity for the GlyR over a GABA$_A$R expressed in the same tissue (170).

5. Tropisetron and other 5-HT$_3$R antagonists

Several 5-HT$_3$R antagonists have potent effects on the GlyR. The most potent of these compounds are those which contain tropeine groups (i.e., esters and amides of 3α-hydroxytropane) and include tropisetron, LY-278,584, zatosetron, and bemesetron. The muscarinic acetylcholine receptor antagonist atropine also belongs in this structural group. Some representative structures are given in Figure 6. This review focuses on tropisetron, the most widely characterized of these compounds.

In 1996, Chesnoy-Marchais found that tropisetron potentiated glycine currents in cultured spinal neurons at low concentrations (0.01–1 μM) but caused inhibition at higher concentrations (66). Its potentiation was mediated by a leftward shift in the glycine dose-response curve. Because the potentiation was additive with the potentiating effects of zinc, ethanol, and propofol (67), tropisetron appeared to act via a novel mechanism. In radioligand binding studies on membrane extracts from the spinal cord and brain stem, tropisetron displaced $[^3$H]strychnine with a $K_i$ of 2 μM (240). In addition, 0.1 μM tropisetron increased the ability of glycine to displace $[^3$H]strychnine (240), suggesting an allosteric enhancing effect on glycine binding. Turning then to an electrophysiological analysis, Maksay et al. (242) subsequently failed to detect tropisetron potentiation in α1- or α2-GlyR homomers or in α1β- or α2β-GlyR heteromers, although the lower apparent affinity inhibitory effect was observed. In apparent contradiction, Supplisson and Chesnoy-Marchais (358) reported tropisetron potentiation in the α1-homeric and the α1β- and α2β-heteromeric GlyRs, but not in the α2-homomer where only inhibition was seen (358). The discrepancy was convincingly resolved by the demonstration that potentiation required a low (EC$_{10}$) glycine concentration, whereas higher (EC$_{50}$) glycine concentrations, as used by Maksay et al. (242), uncovered only inhibition (358). The molecular determinants of tropisetron action have not been elucidated. Because β-subunits are needed to confer tropisetron potentiation to α2-subunits (358), the potentiating binding site may be located at the interface of these subunits.

Tropisetron suppresses glycine-gated currents when applied at high (>10 μM) concentrations to both native neuronal and recombinant GlyRs. At least in the α2-GlyR, it seems to behave in a noncompetitive manner. The tropisetron inhibitory potency is modestly affected (IC$_{50}$ increased by a factor of 2) by the T112A mutation (242), a mutation that completely abolishes the inhibitory potency of zinc. The increased tropisetron inhibitory potency of the α2-GlyR is not transferred to the α1-GlyR via the A2′G mutation (358), the only TM2 domain residue which is not conserved between these subunits. However, as previously seen for CTB and PTX, it may be misleading to infer locations of binding sites on the basis of site-directed mutations at this position in the pore.

A structure-activity study concluded that the tropeine structure itself was required for potentiation (240). However, atropine, which shares the tropeine moiety, does not increase glycine displacement of strychnine binding, although its inhibitory potency is only marginally weaker than that of tropisetron (240, 242). Thus the tropeine moiety appears to be no guarantee of a potentiating effect. These results are broadly consistent with another structure-activity analysis, which concluded that an aromatic ring, a carbonyl group, and a tropeine nitrogen are required for glycineric potentiation (70).

6. Ivermectin

Ivermectin (22,23-dihydroavermectin B$_{1a}$) is a naturally occurring macrocyclic lactone (Fig. 6) that is widely used as an antiparasitic agent in agriculture, veterinary practice, and human medicine (98, 319). Although the target of its antiparasitic action is believed to be a GluClR that exists in a number of invertebrate phyla (78, 178), it also has direct activating or potentiating effects on GABA$_A$Rs and nAChRs (2, 87, 197, 198). At low (0.03 μM)
concentrations, ivermectin potentiates subsaturating glycine responses, but at higher (≥ 0.03 μM) concentrations it irreversibly activates recombinant α1- and α1β-GlyRs (342). Because ivermectin-gated currents have a different pharmacology to glycine-gated currents, and glycine binding site mutations do not drastically affect its sensitivity (342), ivermectin appears to activate the GlyR via a novel mechanism. Apart from cesium (352), ivermectin is the only non-amino acid agonist of the GlyR to be identified to date.

7. Alcohols, anesthetics, and inhaled drugs of abuse

Traditionally, anesthetics have been depicted as non-selective agents that act by partitioning into and disordering lipid bilayers. However, in recent years it has become increasingly apparent that specific binding sites on ligand-gated ion channels are among their major molecular targets (36, 117, 196, 260, 418). Because alcohol and some anesthetic effects on the GlyR are observed at pharmacologically relevant concentrations, it is possible that at least part of their acute effects are mediated by this receptor.

Exposure to pharmacologically relevant (50–100 mM) concentrations of ethanol was first shown by Celentano et al. (59) to produce a persistent increase in the glycine sensitivity of spinal neurons. Most subsequent studies on GlyRs natively expressed in neurons have observed similar potentiating effects. Neonatal ventral tegmental area neurons appear to constitute an exception to this rule: ethanol (0.1–10 mM) potentiated, inhibited, or had no effect on glycine-activated responses in 35, 45, and 20%, respectively, of an impressively large sample of these neurons (422, 423).

Because ethanol potentiation is achieved without disordering the membrane lipid bilayer (366), it is likely to be acting via a specific site at the GlyR. When expressed in Xenopus oocytes, α1-GlyRs were more sensitive to ethanol than were α2-GlyRs (251). This increased sensitivity was abolished by incorporating the naturally occurring spasmodic mouse mutation A52S into the α1-subunit (251). However, the α1-subunit ethanol sensitivity was largely lost upon recombinant expression in mammalian Ltk− or HEK293 cells (380). Because the GlyR α1-subunit contains phosphorylation consensus sites, the phosphorylation status of the receptor was considered a possible cause of this anomaly. Indeed, PKC-mediated phosphorylation selectively increases ethanol potentiation of Xenopus oocyte-expressed α1-GlyRs while having no effect on the enhancement induced by the inhalation anesthetic halothane or the intravenous anesthetic propofol (253). On the other hand, PKA-mediated phosphorylation was without effect (3).

The inhalation (or volatile) anesthetic isoflurane was first shown by Harrison et al. (155) to potentiate recombinant α2-GlyRs. For both recombinant α1-GlyRs and the native GlyR in medullary neurons, the degree of potentiation increased in the rank order methoxyflurane, sevoflurane < halothane, isoflurane, enflurane, F3 (97, 250). The potentiation was induced primarily by a leftward shift in the glycine EC50 (97). Because the leftward shift is more pronounced at low glycine EC values (97), the effects of alcohols and volatile anesthetics have generally been investigated using glycine concentrations that activate 2–5% of the saturating current magnitude.

In contrast to the GlyR, the GABA_C-R is inhibited by both classes of agents (261). By constructing a series of chimeras between these two receptors, a region of 45 amino acid residues was identified as necessary and sufficient for mediating potentiation by both alcohol and volatile anesthetics (262). Site-directed mutagenesis of the nonconserved residues in this region identified S15′ in the TM2 domain and A288 in the TM3 domain as crucial determinants of alcohol and volatile anesthetic sensitivity (262). Because both residues are located towards the extracellular end of their respective TM segments, it was hypothesized that these two residues faced each other to form a pocket to accommodate an alcohol molecule. Lying within the membrane, this putative water-filled pocket is thought to be inaccessible from the ion channel pore. The existence of such a water-filled pocket lined by TM2 and TM3 domains is supported by SCAM data in the GABA_C-R and structural evidence from the nAChR (see sect. ucR).

It was already known that alcohol potentiation of the α1-GlyR increases with the length of the side chain of a series of n-alcohols until a cut-off is reached, after which further increases in molecular size decrease alcohol potency (250). Increasing the size of the amino acid side chain by the S15′Q mutation decreased the cutoff from n = 10–12 to 7, consistent with the expected reduction in the volume of the binding pocket (399, 424). The converse experiment on the GABA_C-R, in which a smaller side chain was introduced at the corresponding position, increased the alcohol size cutoff (399). Using a similar idea, the molecular volume and hydrophathy of the side chain at the 288 position were revealed as crucial determinants of volatile anesthetic sensitivity (420). Together, these experiments supported the view that S15′ and A288 might form the binding pocket for both alcohols and volatile anesthetics. Alternatively, the mutations might impose structural changes that perturb the allosteric potentiation mechanisms of these compounds. Indeed, because these mutations affect glycine EC50 values (262, 420, 424), this alternative is a distinct possibility. More direct evidence for these residues forming a binding pocket came from a SCAM-type approach (252). Current flux through the α1-GlyR incorporating the S15′C mutation was irreversibly enhanced by the sulphydryl-containing anesthetic propa-
directly by propyl methanethiosulfonate. After modification by either compound, the GlyR could no longer be potentiated by alcohols or anesthetics (252). This constitutes strong evidence that these compounds bind specifically in a pocket lined by S15’.

A recent investigation has found that ethanol potentiation of recombinant α1-GlyRs was antagonized by increased atmospheric pressure (85). Because the increased pressure did not affect the actions of glycine, strychnine, or zinc, it is likely to exert a selective effect at the alcohol site. It will be of interest to understand the molecular basis of this phenomenon.

Recently, several commonly abused inhalants were investigated in terms of their action at recombinant α1-GlyRs. Toluene, 1,1,1-trichloroethane, trichloroethylene, and chloroform potentiated α1-GlyRs by reducing the glycine EC_{50} values (31, 33). Because these compounds exhibited different patterns of sensitivity to S15’ mutations than did ethanol and enflurane, and also showed competition with these compounds, it was proposed that their binding sites shared some overlap with the alcohol/anesthetic binding site (31, 33). However, the possibility that the respective binding sites may interact allosterically cannot yet be ruled out.

The gaseous anesthetics nitrous oxide and xenon weakly potentiate submaximal glycine currents in the α1-GlyR. Toluene, 1,1,1-trichloroethane, trichloroethylene, and chloroform potentiated α1-GlyRs by reducing the glycine EC_{50} values (31, 33). Because these compounds exhibited different patterns of sensitivity to S15’ mutations than did ethanol and enflurane, and also showed competition with these compounds, it was proposed that their binding sites shared some overlap with the alcohol/anesthetic binding site (31, 33). However, the possibility that the respective binding sites may interact allosterically cannot yet be ruled out.

The gaseous anesthetics nitrous oxide and xenon weakly potentiate submaximal glycine currents in the α1-GlyR at clinically relevant doses (84, 419). Other anesthetics including propofol, thiopentone, pentobarbitone, alphaxalone (a steroid), etomidate, and ketamine also exert potentiating effects on some GlyR isoforms (35, 84, 250, 295). Because more dramatic effects of all these compounds are seen at other receptors (36, 116, 118, 196, 418), their effects on GlyRs are unlikely to be clinically relevant.

8. Miscellaneous bioactive compounds

Dihydropyridine antagonists of L-type calcium channels also inhibit GlyR currents in spinal cord neurons at micromolar concentrations (69). Their effects are stronger at higher glycine concentrations and increase with time during glycine application, reminiscent of an open channel block mechanism. In addition, low (1–5 μM) concentrations of nitrendipine and nicardipine exert potentiating effects that were additive with those of zinc, implying discrete mechanisms of action (69).

The estrogen receptor modulator tamoxifen has recently been shown to have a particularly dramatic potentiating effect on submaximal glycine responses in cultured spinal neurons (68). A 5 μM concentration caused a 6.6-fold reduction in the glycine EC_{50}. Several controls were used to rule out possible effects of tamoxifen on cell signaling cascades. The magnitude of the potentiation was increased when tamoxifen was applied to the cells before glycine application, although maintenance of the potentiation seen upon glycine application required continued tamoxifen application (68). The potentiation persisted in the presence of 10 μM zinc, implying a discrete site of action. The same study also noted an apparently direct potentiating effect of 4 μM dideoxyforskolin on GlyRs, a finding that may be relevant to studies designed to investigate phosphorylation mechanisms.

Clinical concentrations of the neuroprotective drug riluzole were reported to accelerate the α1β-GlyR desensitization rate while having no effect on the maximal current magnitude (269). Paradoxically, however, the time course of currents activated by brief (2 ms) applications of glycine were prolonged by riluzole (269). Similar effects were also observed on the GABAAR. The prolongation of the simulated synaptic currents may help explain the sedative and anticonvulsant side effects of this drug (95).

Colchicine, a microtubule-depolymerizing reagent, competitively antagonizes glycine-induced currents with IC_{50} values of 64 and 324 μM for the α1- and the α2-GlyRs, respectively. Its effect is instantaneous and independent of the microtubule depolymerization process (238). Interestingly, colchicine also prevents ethanol potentiation of GABAAR currents (394, 397), which perhaps provides a starting point for investigating its mechanism of action.

The tyrosine kinase inhibitor genistein has been shown to have a direct inhibitory effect on native GlyRs in neurons isolated from the hypothalamus and VTA (165, 437). This effect is not a pharmacological effect on PTK activation as it was effective only from the external membrane surface (165). It inhibits in a noncompetitive and use-dependent manner, suggesting that it binds in the pore.

Reasoning that the pharmacology of the GlyR glycine site may have structural similarities with the glutamate (NMDA) receptor glycine site, Schnieden et al. (337) used a potent NMDA receptor glycine antagonist, 2-carboxy-4-hydroxyquinoline, as a lead compound for the development of novel GlyR antagonists. The most potent compound thus identified, 5,7-dichloro-4-hydroxyquinoline-3-carboxylic acid, inhibited recombinant α1-GlyRs with an IC_{50} of 20 μM. Although this compound was not active at the NMDA receptor, the results imply some degree of similarity in the glycine pharmacophores of the GlyR and NMDA receptors. Another glutamate (KA/AMPA) receptor antagonist, NBQX, has recently been shown to inhibit recombinant α1- and α2-GlyRs with an IC_{50} of 4 μM (256).

Effects of ginsenosides, the active ingredients of the Panax ginseng plant, have been investigated on recombinant α1-GlyRs. The most active of these compounds, ginsenoside-Rf, potentiated submaximal glycine-gated currents with an EC_{50} of 50 μM (282).

Glycine-activated currents in recombinant α1- and α2-GlyRs were both inhibited by 3-[2-phosphonomethyl][1,1-biphenyl]-3-yl]alanine (PMBA) with an IC_{50} of
~0.5 μM (163). When applied at higher (10 μM) concentrations, PMBA had differential effects on α2- and α1-GlyRs. In the α1-GlyR, its main effect was to impose a rightward shift on the glycine dose-response. However, in the α2-GlyR, it also lowered the Hill coefficient for glycine activation (163). Furthermore, preincubation with PMBA delayed the rate of glycine-gated current activation in the α2-GlyR but not the α1-GlyR. Thus the mode of PMBA binding or activation may differ between the α1- and α2-subunits.

Opioid alkaloids have long been known to exhibit selectivity for the GlyR over the GABAAR (82). The relative efficacy of these compounds in antagonizing glycine-induced currents in intact spinal neurons is thebaine > morphine > codeine (79, 82), whereas thebaine was by far the most potent of a series of opioid agonists in displacing [3H]strychnine binding in spinal cord neurons, with an IC50 of 1 μM (132).

Finally, as reviewed previously (307), a wide range of compounds with structural similarities to various GABAAR ligands have been investigated in terms of their [3H]strychnine displacement potencies. Benzodiazepines and their antagonists were among the most potent compounds thus identified, with IC50 values of ~1–10 μM (48, 249, 429). Interestingly, a recent report has identified a low-affinity benzodiazepine inhibitory site on α2-containing GlyRs (367).

VII. GLYCINE RECEPTOR CHANNELOPATHIES

A. Human Startle Disease

Human hereditary hyperekplexia, or startle disease, is a rare neurological disorder characterized by an exaggerated response to unexpected stimuli (15, 308). The response is typically accompanied by a temporary but complete muscular rigidity often resulting in an unprotected fall. This behavior is graphically illustrated in a published videotape of the bovine form of the disorder (161). Susceptibility to startle responses is increased by emotional tension, nervousness, fatigue, and the expectation of being frightened. Unprotected falls in turn lead to chronic injuries that are also characteristic of this disorder. Symptoms of this disease are present from birth, with infants also displaying a severe muscular rigidity (or hypertonia) that gradually subsides throughout the first year of life. Some affected infants die suddenly from lapses in respiratory function (129). This disorder has a history of being misdiagnosed as epilepsy, although hyperekplexia is readily distinguished by an absence of fits and a retention of consciousness during startle episodes. The symptoms are successfully treated by benzodiazepines, with clonazepam being the current drug of choice (436).

Hyperekplexia is caused by heritable mutations that reduce the magnitude of glycine-gated chloride currents. As summarized in Table 1, this is achieved by either disrupting GlyR surface expression or by reducing the ability of expressed GlyRs to conduct chloride ions. The disease mutations thereby impair the efficiency of glycnergic neurotransmission in reflex circuits of the spinal cord and brain stem, thus increasing the general level of excitability of motor neurons. Patients are apparently able to cope with this reduced inhibitory tone during normal tasks (perhaps due to developmental compensations, see below), although they are unable to cope with the increased demand required to dampen strong, unex-pected excitatory commands.

Genetic linkage analysis of startle disease pedigrees first localized this disorder to the distal portion of chromosome 5q (327), where the GlyR α1-subunit gene is found. It was subsequently shown that an autosomally dominant form of startle disease was due to either the R271L or R271Q substitutions in the human α1-subunit (346). Although numerous other startle mutations have since been identified, the R271 mutations seem to be the most common cause of this disorder. When incorporated into recombinantly expressed GlyRs, these mutations caused a reduced glycine sensitivity and single-channel conductance (208, 306). Other autosomal dominant startle mutations, including Y279C, K276E, Q266H, and P250T, have generally similar effects (Table 1). In addition, the P250T mutation causes an enhanced desensitization rate (334), which may also contribute to the disease phenotype. As discussed previously in this review, each of these mutations acts by impairing the receptor activation mechanism, and closer analysis of the mutation phenotypes has provided valuable insights into the structural basis of GlyR function. The effect of the autosomally dominant V260M and S270T startle disease mutations (88, 210) has yet to be characterized.

Autosomally recessive forms of startle disease have been described in sporadic cases, generally in the offspring of consanguineous parents. One such patient exhibited homozygosity for a point mutation, I244N (312), in the α1-subunit. When incorporated into recombinant α1-homomeric GlyRs, this mutation resulted in a reduced glycine current magnitude, a reduced glycine sensitivity, and an enhanced desensitization rate (237). Two recessive forms of startle disease have also been described that must have resulted in the complete nonexpression of the GlyR α1-subunit. In one case, the mutation caused a homozygous deletion encompassing exons 1–6 (53), whereas the other involved a base pair deletion resulting in a frameshift and a premature stop codon prior to the TM1 domain (315). Despite the complete absence of α1-subunit expression, the symptoms experienced by these patients were no more severe than in those where the α1-subunit function was only mildly impaired. Another recently identified α1-subunit recessive point mutation, S231R, caused a reduced membrane insertion of functional receptors (166).
TABLE 1. GlyR mutations underlying startle syndromes in various species

<table>
<thead>
<tr>
<th>Mutation and Subunit</th>
<th>Inheritance Mode</th>
<th>Effect on GlyR Function</th>
<th>Reference Nos.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Human forms</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a1 P250T</td>
<td>Autosomal dominant</td>
<td>Reduced single-channel conductance, reduced glycine sensitivity, increased desensitization rate</td>
<td>50, 334</td>
</tr>
<tr>
<td>a1 V260M</td>
<td>Autosomal dominant</td>
<td>As yet unknown</td>
<td>88</td>
</tr>
<tr>
<td>a1 Q260H</td>
<td>Autosomal dominant</td>
<td>Reduced open probability, reduced glycine sensitivity</td>
<td>263, 271</td>
</tr>
<tr>
<td>a1 S270T</td>
<td>Autosomal dominant</td>
<td>As yet unknown</td>
<td>210</td>
</tr>
<tr>
<td>a1 R271LQ</td>
<td>Autosomal dominant</td>
<td>Reduced glycine sensitivity, reduced single-channel conductance</td>
<td>298, 306, 327, 346</td>
</tr>
<tr>
<td>a1 K276E</td>
<td>Autosomal dominant</td>
<td>Reduced glycine sensitivity, reduced open probability</td>
<td>101, 228, 237</td>
</tr>
<tr>
<td>a1 Y279C</td>
<td>Autosomal dominant</td>
<td>Reduced glycine sensitivity, reduced whole cell current magnitude</td>
<td>205, 237, 345</td>
</tr>
<tr>
<td>a1 I244N</td>
<td>Autosomal recessive</td>
<td>Reduced glycine sensitivity, reduced whole cell current magnitude, increased desensitization rate</td>
<td>237, 312</td>
</tr>
<tr>
<td>a1 Deletion of exons 1-6</td>
<td>Autosomal recessive</td>
<td>Presumed nonfunctional</td>
<td>53</td>
</tr>
<tr>
<td>a1 S231R</td>
<td>Autosomal recessive</td>
<td>Reduced membrane insertion</td>
<td>166</td>
</tr>
<tr>
<td>a1 Stop codon at Y202</td>
<td>Autosomal recessive</td>
<td>Reduced surface expression, possible heterozygosity with a1 V147M</td>
<td>315</td>
</tr>
<tr>
<td>a1 G342S</td>
<td>Compound heterozygous?</td>
<td>No effect of individual mutation, possible heterozygosity with other mutations</td>
<td>315</td>
</tr>
<tr>
<td>a1 R253H + a1 R392H</td>
<td>Compound heterozygous</td>
<td>Reduced membrane insertion</td>
<td>311, 384</td>
</tr>
<tr>
<td>β G229D + β exon 5 loss</td>
<td>Compound heterozygous</td>
<td>Reduced glycine sensitivity, reduced surface expression</td>
<td>314</td>
</tr>
<tr>
<td><strong>Murine forms</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β Line-1 intronic insertion</td>
<td>Autosomal recessive (Spastic)</td>
<td>Reduced surface expression</td>
<td>156, 187, 275</td>
</tr>
<tr>
<td>a1 A52S</td>
<td>Autosomal recessive (Spasmodic)</td>
<td>Reduced glycine sensitivity</td>
<td>335</td>
</tr>
<tr>
<td>a1 Stop codon</td>
<td>Autosomal recessive (Oscillator)</td>
<td>Reduced surface expression</td>
<td>54</td>
</tr>
<tr>
<td><strong>Bovine form</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a1 Stop codon</td>
<td>Autosomal recessive (Myoclonus)</td>
<td>Reduced surface expression</td>
<td>147, 294</td>
</tr>
</tbody>
</table>

Compound heterozygous forms of startle disease have also been described. In one case, two distinct recessive a1-subunit mutations, R252H and R392H, caused startle disease when present in different alleles (384). However, patients possessing either single mutant allele were healthy. Consistent with this observation, coexpression of the two mutant subunits resulted in a reduced surface expression of functional GlyRs, whereas the individual mutations had no effect (311). The recessive M147V and G342S a1-subunit mutations have also been identified in patients exhibiting startle disease symptoms (315). These mutations are presumed to result in compound heterozygous forms of startle disease, but if this is the case their partner mutations remain to be identified. Finally, compound heterozygous mutations have recently been identified in the β-subunit. A β-subunit missense mutation (G229D) and a splice site mutation (resulting in the excision of exon 5) occurred simultaneously in a compound heterozygote with a transient startle disease phenotype (314). The G229D mutation alone induced a modest (4-fold) decrease in the glycine sensitivity of recombinant α1β-GlyRs (314), whereas the removal of exon 5 would presumably have precluded the expression of functional β-subunits.

To date, no startle mutations have been identified in the human α2- or α3-subunits. It remains to be determined for any species whether expression of these subunits is upregulated to compensate for the loss of α1-subunit expression or function. The effectiveness of clonazepam in treating startle disease is presumably due to its potentiation of the GABA<sub>A</sub>R. Indeed, evidence from spastic mice (26) (135) and myoclonic cattle (233) suggests that spinal cord GABAergic neurotransmission may be upregulated during development in compensation for the loss of glycineric tone.

It would not be surprising if startle syndromes resulted from mutations that disrupt the function of other proteins involved in the formation, maintenance, and function of glycineric synapses. Indeed, a hereditary mutation in the GlyR clustering protein gephyrin results in a hyperekplexia phenotype in humans (313) and targeted deletion of the glycine transporter subtype 2 gene produces a startle phenotype in mice (133).

**B. Murine Startle Syndromes**

Three naturally occurring murine startle syndromes have been identified to date (Table 1). The symptoms of
each are largely similar to those observed in human hyperekplexia (308). An exception is that mouse startle symptoms commence at around the 20th postnatal day, corresponding to the time when the replacement of α2-homomers by α1β-heteromers is complete. The most thoroughly characterized murine syndrome is the spastic mouse, which was first identified in the 1960s (255). This autosomal recessive disorder is characterized by a reduction in the number of expressed GlyR α1-subunits, although the function of the expressed receptors is normal (26, 28). The spastic phenotype was found to be caused by the insertion of a 7.1 kb Line-1 repeating element into intron 5 of the β-subunit (187, 275). This element leads to aberrant splicing of the β-subunit transcripts resulting in an accumulation of prematurely terminated protein and a concomitant reduction in the surface expression of functional GlyRs (53, 294, 315). A possible reason for the difference is that the α2 → α1β subunit switch in the mouse occurs after birth resulting in an inability to feed. In humans and cattle, the switch is likely to occur before birth, thereby not affecting the ability to feed, and affording time for the development of synaptic adaptations before parturition.

Two transgenic mice strains have been generated with a view to developing an animal model of human hyperekplexia. The first approach mimicked the spastic mouse mutation by engineering a transposon insertion into the β-subunit (29). Homozygous mice displayed a startle phenotype that resembled human hyperekplexia (30). The second approach incorporated the dominant human α1-subunit R271Q mutation into transgenic mice. Transgenic mice that were heterozygous for the R271Q mutation displayed a pronounced startle phenotype, and mice homozygous for the mutation were not viable (30). Knock-in mice bearing the α1-subunit S267Q mutation (which eliminates the alcohol binding site and reduces peak current magnitude) displayed a reduced sensitivity to alcohol and an oscillator-type phenotype (111, 112). Finally, as noted above, knock-out of the glycine transporter subtype-2 gene produces a lethal oscillator-type phenotype (133).

C. Bovine Myoclonus

A congenital recessive startle syndrome, called myoclonus, has been identified in Poll Hereford cattle (160). This disorder was recently shown to be due to a single base pair deletion in the α1-subunit gene, leading to a frameshift and a premature stop codon before TM1 (294). As would be expected, this mutation induced a dramatic reduction in the surface expression of functional GlyRs (147). A similar syndrome may also exist in Peruvian Paso horses (148).

VIII. OUTLOOK

GlyRs have important roles in a variety of physiological processes, especially in mediating inhibitory neurotransmission in the spinal cord and brain stem. Although recent progress in understanding the molecular functional architecture of these receptors has been rapid, many gaps remain in a number of critical areas. The
following are considered to be among the most pressing research priorities at the present time.

1) A high-resolution structure of the entire GlyR complex will permit the design of much more precise experiments to understand receptor structure and function. Unfortunately, however, crystal structures are notoriously difficult to obtain for membrane-spanning proteins, and complete structures have yet to be resolved for any LGIC member.

2) It is essential to establish the exposure pattern of residues in TM2 and in the pore selectivity filter of the GlyR α- and β-subunits. This is a prerequisite for understanding the ion-permeation and channel-opening mechanisms. It is also essential to probe the surface electrostatic potential in the pore selectivity filter to further our understanding of the ionic charge discrimination mechanism.

3) Because binding sites are located at subunit interfaces, it is necessary to resolve the GlyR subunit stoichiometry and arrangement so that the number and type of subunit interfaces can be defined. This is an important first step toward resolving the structural and functional basis of ligand binding.

4) There are large gaps in our knowledge of glycine binding mechanisms. In particular, the role of the α-subunit complementary binding domains in coordinating glycine needs to be investigated. This is a prerequisite for the structural modeling of the agonist binding pocket.

5) The role of β-subunit in channel agonist binding and channel activation has received scant attention. Again, this information is necessary for the structural modeling of ligand binding sites and the understanding of activation mechanisms.

6) The modeling of GlyR kinetics remains controversial. Because kinetic models are crucial for understanding and predicting receptor behavior, it is hoped that future studies will carefully readdress this situation.

7) The diversity of GlyR subtypes at least partially underlies the diversity in glycine neurotransmission properties throughout the central nervous system (219). Understanding the GlyR structural variations that underlie this synaptic functional diversity is an important question for future research.

8) The role of α2-homomeric GlyRs in embryonic neurons remains to be clarified.

9) Extrasynaptic GlyRs are present on many central nervous system neurons. In addition, GlyRs have been found in a number of nonneuronal tissues. The physiological roles of these GlyRs need to be investigated in more detail.

10) The therapeutic possibilities of GlyR modulatory agents warrant further investigation. As noted in a recent review (215), the fact that GlyRs are involved in motor reflex circuits and nociceptive sensory pathways suggests that GlyR modulators could have therapeutic potential as analgesics and muscle relaxants. This review describes the effects of a number of molecules with potential to be lead compounds for the development of such therapeutics. Further therapeutic possibilities may emerge as the roles of extrasynaptic and nonneuronal GlyRs are characterized in more detail.

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