STRUCTURE AND FUNCTION OF GLUTAMATE RECEPTOR ION CHANNELS

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Abstract  A vast number of proteins are involved in synaptic function. Many have been cloned and their functional role defined with varying degrees of success, but their number and complexity currently defy any molecular understanding of the physiology of synapses. A beacon of success in this medieval era of synaptic biology is an emerging understanding of the mechanisms underlying the activity of the neurotransmitter receptors for glutamate. Largely as a result of structural studies performed in the past three years we now have a mechanistic explanation for the activation of channel gating by agonists and partial agonists; the process of desensitization, and its block by allosteric modulators, is also mostly explained; and the basis of receptor subtype selectivity is emerging with clarity as more and more structures are solved. In the space of months we have gone from cartoons of postulated mechanisms to hard fact. It is anticipated that this level of understanding will emerge for other synaptic proteins in the coming decade.

INTRODUCTION

The amino acid S-glutamate acts as the neurotransmitter at the majority of excitatory synapses in the brain and spinal cord of vertebrates. The pioneering work establishing the physiological role of glutamate receptor ion channels (iGluRs) in synaptic transmission relied heavily on the development of selective agonists and antagonists. By 1980 this approach had led to the recognition of three major iGluR subtypes, the AMPA, kainate, and NMDA receptors, which were named after their selective agonists (1). Each of these receptors activates a cation-selective ion...
channel permeable to Na$^+$ and K$^+$, with differing degrees of permeability to, and
block by, the divalent cations Ca$^{2+}$ and Mg$^{2+}$ (2). This functional classification
remains in widespread use and indeed largely defines the biology of glutamate
receptor ion channels.

A corresponding molecular classification of glutamate receptor channels arose
from the application of cDNA cloning techniques. This revealed that each of the
major functional subtypes of glutamate receptor ion channel was comprised of a
family of genes. The AMPA receptors are assembled from the GluR1-GluR4 sub-
units (also called GluRA-GluRD). Two gene families, GluR5-GluR7 and KA1/
KA2, encode kainate receptors; the latter form functional channels only when
coaessembled with members of the GluR5-GluR7 family. NMDA receptors, which
typically must bind both glutamate and glycine for activation, are assembled from
three gene families. All NMDA receptors contain obligate NR1 subunits that serve
two roles. The NR1 subunits contain the glycine-binding site, but in addition are
required for trafficking of heteromeric receptor assemblies to the plasma membrane.
NR1 subunits coassemble with the NR2A-NR2D subunits that encode glutamate-
binding sites; less commonly they coassemble with the NR3A/NR3B gene families
that encode glycine-binding sites distinct from those on the NR1 subunit. Two or-
phan members of the iGluR gene family, the delta 1 and delta 2 receptors, are not
well characterized because their functional activity and ligand-binding properties
have defied analysis. Adding further complexity to the functional analysis of the
iGluR gene family is the occurrence of alternative splicing and RNA editing. Fi-
ally, the ongoing sequencing of microbial genomes has resulted in the discovery
of a growing family of bacterial ligand–gated ion channels of which GluR0 was the
first to be characterized (3, 4). Several reviews summarize much of the above mate-
rial and should be consulted for in-depth discussion of the physiological roles and
molecular biology of glutamate receptor ion channels and their roles in synaptic
function (4–9).

Domain Organization of Glutamate Receptor Ion Channels

The cloning of iGluR genes led immediately to attempts to relate their amino acid
sequence to function and mechanism. Progress was at first limited by the absence
of structural information, and for several years experiments were designed and
interpreted on the basis of an erroneous transmembrane topology model derived
from one for ligand-gated nicotinic receptor ion channels. The recognition that
iGluRs had a unique topology was coupled to the discovery that their N-terminal
and agonist-binding domains shared amino acid sequence homology with bacterial
periplasmic-binding proteins and were thus likely to have a similar structure. The
periplasmic protein homology–based model for the agonist-binding domain was,
however, incompatible with the topology present in nicotinic receptor ion chan-
nels. Establishment of the correct iGluR topology was a major step forward and
paved the way for a direct analysis of iGluR structure and function. Such analysis
revealed that iGluRs are composed of an agonist-binding domain interrupted by insertion of a two-transmembrane (TM) pore loop ion channel; in eukaryotic but not prokaryotic iGluRs there is an additional N-terminal domain, a third TM segment, and a cytoplasmic C-terminal domain (Figure 1). In some fish, amphibian, and bird iGluRs the N-terminal domain is absent.

**AGONIST-BINDING CORE** Key experiments, which defined the correct TM topology for iGluRs, combined two approaches. The first was genetic insertion of glycosylation and proteolytic enzyme recognition sites, followed by expression in eukaryotic cells and analysis of shifts in molecular weight produced by incubation with glycosidases and proteases. This approach, combined with hydropathy analysis, defined the boundaries of the extracellular and, by inference, intracellular and TM polypeptide segments (10–13). The second approach capitalized on this revised topology and utilized homology modeling based on the hypothesis that the agonist-binding domain of iGluRs was likely to be a two-domain structure similar to that in periplasmic-binding proteins, the structures of several of which had already been solved (14–16). Although the sequence homology of iGluRs to periplasmic proteins was recognized early on (17), interruption of the homologous region by insertion of the ion channel pore domain hindered recognition that the agonist-binding core recognizes ligands by a mechanism similar to that in periplasmic proteins. Once the correct topology for iGluRs was established, it was possible to convert the agonist selectivity of an acceptor protein by swapping the S1 and S2 segments between AMPA and kainate receptor subunits. This proved that in combination the S1S2 segments constituted the agonist-binding core despite the insertion of the ion channel domain (15). Further homology modeling, combined with site-directed mutagenesis, reinforced the conclusion that the S1S2 segments formed the agonist-binding site in AMPA, kainate, and NMDA receptors and that their structure was related to that in periplasmic proteins (18–20). Such studies paved the way for the establishment of the domain organization of an iGluR subunit (illustrated in Figure 1), in which the agonist-binding core is a discrete structure, joined to the N-terminal domain and the ion channel pore by polypeptide linkers of currently unknown structure. It is now widely accepted that four subunits assemble to form functional iGluRs, although direct experimental proof for this has yet to be obtained.

**AMINO-TERMINAL DOMAIN** The ∼400-amino acid polypeptide segment that makes up the N-terminal domain is a major, but not sole, determinant of subtype-specific assembly within iGluR gene families (21, 22). In the case of the AMPA receptor GluR4 and NR1 subunits, the N-terminal domain forms dimers in solution (22, 23), most likely related to a role in receptor assembly. Homology modeling and functional tests by site-directed mutagenesis established that this domain has a fold similar to that of leucine-isoleucine-valine-binding protein (LIVBP) and the agonist-binding domain in G-protein–coupled glutamate receptors (mGluRs). The
structure of the N-terminal domain is expected to be distinct from the structure of the agonist-binding core of iGluRs because in LIVBP and mGluRs, the two globular domains are connected by three β-strands instead of the pair of β-strands found in the iGluR glutamate- and glycine-binding cores (24, 25). The two-domain fold of the N-terminal domain suggests that it might also encode a ligand-binding site, but identification of the endogenous ligands, if any, for the majority of iGluR N-terminal domains has yet to be attempted. In the case of NMDA receptors, functional experiments have established that Zn2+ and the noncompetitive antagonist ifenprodil bind to the N-terminal domain to modulate ion channel gating via an allosteric mechanism (26–29). The details of the coupling mechanism remain obscure, in part, because the structure of the N-terminal domain and its packing relative to the other domains in iGluRs remain to be determined.

ION CHANNEL PORE The S1 and S2 polypeptide sequences that make up the agonist-binding core are interrupted by insertion of two, membrane-spanning, most likely α-helical segments, which in combination with a pore helix and pore loop make the narrowest part of the pore (3, 30–32). Although the ion selectivity of eukaryotic iGluRs and K+ channels is strikingly different, they share significant amino acid sequence homology and probably have similar structures (3, 4, 30). Indeed, in the case of some of the recently discovered bacterial iGluRs, which have the required amino acid sequences to selectively bind K+ but not Na+, the structure of the selectivity filter is almost certainly like that determined for the potassium channels KcsA, MthK, and KvAP (33–35).

Because the structural basis for K+ selectivity requires a rigid shell of main chain carbonyl oxygen atoms in the pore loop that replace water molecules of solvation during passage of K+ ions through the lipid bilayer (36), it is easy to imagine either of two scenarios that would allow nonselective permeation of Na+ and K+ through a similar structure in iGluRs. Either the pore is sufficiently wide to allow Na+ and K+ ions to pass the narrowest segment in their hydrated state, or the pore loops in iGluRs are more flexible than those in K+ channels, thus permitting close contact with both Na+ and K+ ions during permeation. Measurement of the permeability of large organic cations supports the former mechanism (37, 38). It is also conceivable that in iGluRs the narrowest part of the pore is partly lined by amino acid side chains rather than main chain peptide bonds as occurs in K+ channels (33, 36), but determining this will require structural analysis.

In the case of bacterial iGluRs, the S2 segment of the agonist-binding domain is followed by a short extracellular C terminus (3, 39), whereas in vertebrate iGluRs, the S2 segment is linked to a third TM segment such that the C terminus is intracellular (Figure 1). The functional role of the last TM domain has not been clearly defined, but it appears, at least for NMDA receptors, to participate in channel gating because mutations in this region alter desensitization rates as well as channel open times (40). Mutants lacking this domain cannot form functional receptors (22, 41). Interestingly, expression of the deleted domain in trans is sufficient to rescue channel activity (41).
CARBOXYL-TERMINAL DOMAIN  The C terminus of vertebrate iGluRs varies in length from around 20 to 500 amino acids. It interacts with numerous cytoskeletal proteins and is important for receptor trafficking (42, 43). The C-terminal domain in NMDA receptors is much larger than that in AMPA and kainate receptors, suggesting that it might have as yet unknown functions. Amino acid sequence alignments do not reveal significant homology with other proteins, and direct structural studies will be required to make progress in understanding the role of this domain.

EXPRESSION AND CRYSTALLIZATION OF AGONIST-BINDING CORES

The discovery that the agonist-binding core of iGluRs is a two-domain structure, interrupted by insertion of the membrane-spanning ion channel pore, led to the realization that with protein engineering it should be possible to genetically excise the agonist-binding domain from the pore region and generate a water-soluble protein that retains ligand-binding activity. Overexpression of constructs in which the agonist-binding core encoded by the S1 and S2 segments was isolated from the N-terminal domain, pore region, and C-terminal domain permitted for the first time biochemical and subsequently structural approaches to be applied to the study of iGluR function. This approach should eventually permit a similar analysis of the N- and C-terminal domain structures, although progress to date has been slower than for the agonist-binding core.

The initial S1S2 constructs generated for the AMPA receptor GluR4 subunit established that the isolated domain retained the appropriate selectivity for AMPA receptor-preferring ligands, which bound with $K_d$ values similar to those established for full-length receptors in membranes (44). Extensions of this approach subsequently allowed the successful expression of the agonist-binding cores for GluR2 (45), GluR6 (46), and NR1 (47, 48).

These initial attempts at over-expression of iGluR agonist-binding cores, while successful in establishing the feasibility of the approach, needed further modification before structural studies were possible. First, protein expression levels, which were on the order of 0.1 mg S1S2 protein per liter of cell culture in the initial studies on AMPA receptors, were increased 10- to 100-fold to yield sufficient protein for attempts at crystallization. This was achieved by establishing conditions for the refolding of milligram quantities of denatured GluR2 S1S2 protein expressed in *Escherichia coli* and purified from inclusion bodies (49). More recently, strains of *E. coli* engineered for expression of proteins containing disulfide bonds have allowed expression of milligram quantities of iGluR S1S2 constructs without the need for refolding (50). Second, to obtain well-ordered crystals that diffract to high resolution it was necessary to define the minimum S1S2 constructs that retained biological activity and conformational stability. Such protein engineering is typically required in crystallographic studies because the flexible polypeptide segments present at the N and C termini, and in loops extending from the core of
the protein, typically adopt a range of conformations that interferes with the packing required to generate the well-ordered crystal lattice needed for high-resolution diffraction of X rays. Starting from the initial constructs that were first reported to retain AMPA-binding activity, a series of nine additional GluR2 constructs was generated and screened for binding activity and agonist-induced resistance to proteolysis. This led to the identification of a first generation construct, which when crystallized with kainate, diffracted to 1.5 Å using synchrotron radiation (51). On the basis of this approach the agonist-binding cores have been crystallized for GluR0 (39), GluR2 (52), GluR6 (M.L. Mayer, unpublished work), and NR1 (50). The similar fold of the S1S2-binding cores for GluR0 and for AMPA, kainate, and NMDA receptors, with the agonist bound in a cleft between domain 1 and domain 2, is shown in Figure 2 and illustrates the contributions of the S1 and S2 peptide segments to each domain.

STRUCTURAL STUDIES ON AMPA RECEPTORS

In initial experiments with the first generation GluR2 S1S2 construct, complexes with glutamate and other ligands did not give crystals with sufficient diffraction for structure determination (52). However, after a further round of protein engineering to remove disordered peptide sequences, for which electron density was missing in the first generation complex with kainate, a second generation construct was obtained that crystallized both in the ligand-free (apo) state as well as with a range of agonists and antagonists (53). At present this construct has been used to solve the crystal structures of GluR2 S1S2 complexes with 12 full and partial agonists, 2 competitive antagonists as well as some allosteric modulators, and the apo state. Complementing these static structures are studies of the protein dynamics of glutamate-bound GluR2 S1S2 (54). The use of protein engineering to obtain a well-ordered protein core, as well as expression in bacteria to prevent glycosylation, which frequently produces disorder in protein crystals, was a key determinant in the success of this strategy, and when this was not done, the crystals obtained did not diffract to high resolution (55). Studies with GluR4 do not reveal any difference in ligand binding, function, stability, or cell surface expression of protein in which S1S2 is either glycosylated or nonglycosylated (56).

The first crystal structure of the GluR2 S1S2 kainate complex revealed a two-domain globular protein with the agonist partially buried in a cleft between the two lobes (52). The lobes are connected by a pair of antiparallel β-strands, and the overall structure is similar to the fold of GlnBP, the periplasmic-binding protein for glutamine (57). The larger of the domains is encoded by the peptide segment S1 but includes two α-helices and a β-strand that occur after the ion channel insertion and are thus encoded by S2. This was an important finding because it showed that the S1 and S2 peptide segments do not correspond to discrete structural domains within the agonist-binding core. Also of note was the observation that the extent of domain closure of the kainate complex was intermediate between that for the
apo and glutamine-bound forms of GlnBP. The significance of this for determining
the mechanism of partial agonism became apparent when structures of complexes
with glutamate and other full agonists were solved.

**MECHANISM OF ACTIVATION BY AGONISTS**

Subsequent structural studies on GluR2 S1S2, using the more readily crystallized
second generation construct, established that, similar to its periplasmic counter-
parts, GluR2 S1S2 undergoes a large conformational change upon binding ag-
onists, switching from a relaxed, open-cleft apo conformation, to a constrained
closed-cleft conformation when bound by AMPA and glutamate (53). Compared
with the open-cleft apo structure, the lobes of the AMPA and glutamate complexes
rotate closer together by $\sim 21^\circ$. Both X-ray crystallographic (58) and biochemical
investigations (59) suggest that the mechanism for agonist binding and domain
closure is a two-step process. In the docking step, the agonist binds to residues in
domain 1 via its $\alpha$-amino and $\alpha$-carboxyl groups. With the agonist partially secured
in the binding cleft, the $\gamma$-carboxyl group electrostatically attracts the base of helix
F that points its N-terminal dipole into the cleft. The locking step involves rotation
of domain 2 toward domain 1 and closure of the binding cleft. How this domain
closure might open the ion channel was revealed by the observation that in the
majority of crystal forms studied, the agonist-binding cores form dimers in which
the agonist-binding clefts face outward, and the two S1S2 linkers, which replace
the ion channel pore, lie on the same side of the dimer on the face opposite from the
N-terminal domain. The dimer surface, which buries 1150 $\AA^2$ of solvent-accessible
surface per monomer, is formed exclusively by domain 1. As a result, when
the pair of subunits in a dimeric assembly undergoes agonist-induced stabilization
of the closed cleft conformation, the pair of ion channel linkers on domain 2 swing
apart from each other, like a pair of opening scissors. Because these linkers are
replacing the channel-forming region from the full-length receptor, it is easy to
envision how agonist-induced domain closure can do work on the TM segments,
pulling or twisting the ion channel to open (Figure 3).

The dimer interface is a unique feature of iGluRs. Both GluR2 S1S2 and its
bacterial homologue GluR0 S1S2 assemble as dimers in solution and crystal-
lize as dimers, whereas the bacterial periplasmic-binding proteins do not. Amino
acid sequence alignments for the agonist-binding cores of GluR0, GluR2, and
three structurally related periplasmic proteins, reveal conservation of hydropho-
bic residues in the GluR0 and GluR2 dimer interface, whereas the corresponding
surface of periplasmic proteins contains charged amino acid residues that form
noncomplementary contacts when two monomers are superimposed on the GluR0
dimer (39). Thus despite folds similar to those of periplasmic proteins, the
surface of the ligand-binding core of iGluRs has evolved to support the formation
dimeric assemblies that play a key role in activation, and desensitization (see
below).
Recent crystallographic studies on the MthK calcium-activated potassium channel led to the proposal of a gating mechanism in which ligand-induced closing and rotation of a set of four two-domain calcium-binding cores produced a 30° bend in the inner TM helix thus opening the pore (34, 60). A conserved glycine residue in the inner helix of MthK acts as a hinge that permits this motion and is conserved in other K⁺ channels. Because GluR0 is also a two TM ligand–gated K⁺ channel, a gating mechanism identical to that in MthK would have been expected. However, sequence alignments show that for GluR0 and related prokaryotic iGluRs K⁺-selective channels the glycine hinge has been replaced by an alanine or serine, possibly because the hinge has moved closer to the start of M2. In this context it is interesting that in inward rectifier K⁺ channels the glycine hinge also has moved and appears to be closer to the C terminus of the inner helix than in MthK. Curiously, for three of the prokaryotic nonselective iGluRs that lack the TVYGYD signature sequence, there is a glycine at the MthK hinge position. Eukaryotic iGluRs contain numerous conserved glycine residues in their TM segments, the location of which differs for individual iGluR subtypes (Figure 4). It is likely that at least some of these glycine residues act as hinge regions, similar to the mechanism proposed for MthK, and that several helices must bend during gating, as suggested by the glycine-rich sequence of M3.

THE MECHANISM OF ACTION OF COMPETITIVE ANTAGONISTS

According to classical receptor theory, competitive antagonists bind to the agonist recognition site but do not activate the receptor. Many iGluR competitive antagonists are larger than their agonist counterparts, and thus a likely mechanism for antagonism would be steric interference with the activation process. Crystal structures of complexes of the GluR2 S1S2 agonist-binding core with two different antagonists, DNQX and ATPO, reveal just such a mechanism. Even though DNQX and ATPO have entirely unrelated chemical structures, the former is a quinoxalinedione and the latter is from the isoxazole family of compounds, they both appear to prevent domain closure via a foot-in-the-door mechanism (53, 61). However, as their different chemical structures suggest, the location of the foot is different. For DNQX, it is probably the interaction between the 7-nitro group and Thr686 in domain 2 that props the binding cleft open. Interestingly, ATPO, which is a derivative of AMPA, interacts with the same subsites as conventional agonists. In this case, the foot, or the 3-phosphono-methoxy group, binds to the base of helix F; agonists interact with the same location in domain 2. The extended distance between the isoxazole ring and the phosphono-methoxy group is what prevents domain closure with ATPO in the binding cleft. Of note, DNQX crystals were grown in a buffer containing ammonium sulfate, and in the receptor antagonist complex a sulfate ion acts as a bridge between the base of helix F and the antagonist molecule. Whether the binding of quinoxalines such as CNQX and...
DNQX is altered by sulfate or phosphate ions in vivo has not been determined, and it is possible that a network of water molecules can support the binding of these antagonists in the absence of sulfate or phosphate. Also of interest is the finding that compared with the apo structure, both DNQX and ATPO produce between 2.5 and 6.0° of domain closure. In wild-type receptors this is insufficient to activate ion channel gating, but in iGluRs with the lurcher mutation, which destabilizes the closed state of the channel (62), quinoxalines act as partial agonists (63).

THE BINDING SITE FOR AMPA RECEPTOR-SELECTIVE AGONISTS

The GluR2 S1S2 construct used for crystallographic studies is a member of the AMPA receptor gene family for which a large series of ligands has been synthesized. At present, structures have been solved for the agonists glutamate, AMPA, and kainate (52, 53); quisqualate (64); willardiine and its 5-F, 5-Br, and 5-I derivatives (65); and a series of isoxazoles, (S)-2-Me-Tet-AMPA, (S)-ACPA, (S)-Br-HIBO (66), and ATPA (67). A key feature that emerged from these studies was the demonstration that in the active closed cleft conformation, the GluR2 agonist-binding pocket is sufficiently large to accommodate three water molecules as well as glutamate. In combination with agonists, these water molecules supply surrogate ligand atoms and contribute to a hydrogen bond network holding the agonist-binding core in its active, closed cleft conformation. Displacement of subsets of these structural water molecules allows the binding of heterocyclic glutamate analogues such as AMPA and its derivatives, quisqualate and the willardiines. Analysis of these structures also revealed that not all AMPA receptor–selective agonists act as isosteres of glutamate. A map of the occupancy of subsites within the agonist-binding pocket by individual agonists is shown in Figure 5. This reveals that quisqualate and the willardiines have interactions identical to those for glutamate, whereas for some of the sites occupied by AMPA and kainate, there is an interchange between water molecules and ligand atoms.

STRUCTURAL BASIS FOR PARTIAL AGONISM

Agonist efficacy, or the ability to activate once bound, is defined by the unique interactions that each agonist makes with the receptor. As discussed above, AMPA and glutamate both induce ~20° domain closure in GluR2 S1S2, and both agonists evoke similar size currents from non-desensitizing GluR2 receptors expressed in Xenopus oocytes (58). The AMPA and glutamate structures are essentially identical to each other, as well as to structures solved in complex with other full agonists such as quisqualate (65), ACPA, and 2-Me-Tet-AMPA (66). However, as shown in Figure 5, all these agonists occupy distinct combinations of subsites within the binding cleft and have unique contributions from water molecules. This suggests...
that the common conformation of the binding cleft is what defines the activity of these agonists. Indeed, the S1S2 complex with the partial agonist kainate has a conformation unlike that of the full-agonist structures: The GluR2 S1S2 kainate complex shows only 12° of domain closure relative to the apo conformation. The intermediate degree of domain closure produced by kainate, coupled with the observation that kainate also acts as a partial agonist at native AMPA receptors (68), suggests that the extent of domain closure determines the strength of ion channel activation. Although appealing, this hypothesis suffered from a number of complications, including the observation that glutamate and kainate bind to different subites in the agonist-binding core and that only a single pair of ligands was compared. In addition, it remained unclear how differences in domain closure are related to ion channel activity. Reinforcement for the hypothesis that the extent of domain closure is related to the degree of activation by AMPA receptor agonists came from studies of a series of heterocyclic compounds related in structure to AMPA (66). One of these, (S)-Br-HIBO, produced 18° domain closure and generated 85% of the maximum response evoked by glutamate, whereas the other compounds examined, AMPA, 2-Me-tet-AMPA, and ACPA, produced the same extent of domain closure as glutamate and acted as full agonists.

In an attempt to address some of these issues, two sets of experiments were performed. The first utilized the high-resolution structure of the GluR2 S1S2 kainate complex, careful examination of which revealed that the isopropenyl group of kainate prevented full domain closure owing to collision with the side chains of Tyr450 and Leu650 (52, 53). Mutation of the hydrophobic leucine side chain to the smaller polar threonine side chain was expected to permit further domain closure and increase the efficacy of kainate. The L650T mutant behaved as predicted and increased the efficacy of kainate from 2 to 24% relative to maximum response for the reference compounds glutamate and quisqualate, which behave as full agonists in both wild-type GluR2 and the L650T mutant (58). The structure of the L650T S1S2 complex with kainate revealed a corresponding increase in domain closure from 12° for the wild-type complex to 15° for the L650T mutant complex.

An unexpected result of these experiments was the observation that AMPA, one of the reference compounds that acts as a full agonist on wild-type AMPA receptors (with efficacy similar to glutamate and quisqualate) behaved as a partial agonist for the L650T mutant with a maximum response only 38% of that produced by glutamate and quisqualate. Crystal structures of the L650T mutant S1S2 complex with quisqualate and AMPA were solved and revealed no difference from wild-type for quisqualate, whereas for AMPA, the agonist-binding core adopted both fully closed and partially closed conformations. Destabilization of the fully closed AMPA-bound conformation probably results from reduced hydrophobic contacts between the face of domain 1 with the Leu650 side chain in domain 2 and perturbation of solvent structure in the AMPA-bound complex.

The observation that the AMPA-bound complex of the L650T mutant can adopt multiple conformations suggests that the agonist-binding core has greater conformational flexibility than previously anticipated and that the degree of domain
GLUTAMATE RECEPTOR ION CHANNELS

Closure is a direct determinant of agonist efficacy. Nonetheless, because the mode of binding of AMPA and kainate differs from that for glutamate and quisqualate, it was desirable to perform similar experiments using a series of structurally related partial agonists that bind to the same sites as glutamate. The 5-substituted willardiines, which bind exactly like glutamate and act as partial agonists at AMPA receptors, were ideal compounds for this purpose. The willardiines act as partial agonists because close contacts between the 5-substituent of the uracil ring and the side chain of Met708 prevent complete domain closure. For glutamate and the willardiine five-position series—H, F, Br, and I—Met708 adopts one of three different rotamers as the size of the ligand increases, but because this is insufficient to fully relieve steric hindrance, there is an additional rigid body movement of domain 2, allowing the agonist-binding core to adopt a progressively more open conformation. Coupled to this variation in the extent of domain closure for the GluR2 S1S2 agonist-binding core is a corresponding reduction in the extent of activation and desensitization of the full-length receptor as the size of the 5-substituent is increased (69).

Viewed in the context of the GluR2 S1S2 dimer, domain closure leads to an increase in the distance between protomer linker regions. Because these linker regions replace the channel-forming region from the full-length receptor, linker separation not only suggests a physical mechanism for channel activation but also reveals, when plotted against agonist efficacy, how incremental increases in domain closure lead to corresponding increases in ion channel activation. Summarized in Figure 6 is the correlation between linker separation and relative agonist efficacy for all the crystal structures for which there is corresponding functional data. The relatively linear correlation for the agonist-bound points is striking and provides strong support for the initial domain closure hypothesis. The major anomaly on this plot, 5-iodo-willardiine, has a distorted linker because of crystal lattice contacts in this region; however, consistent with the trend for other AMPA receptor partial agonists, the degree of domain closure measured for 5-iodo-willardiine was intermediate between that for kainate and 5-bromo-willardiine (69). Such a trend does not exclude the existence of additional mechanisms for partial agonism as seen, for example, in the L650T complex with AMPA, which alters the stability of the closed cleft conformation such that on average partial agonist-bound receptors enter this state less frequently than full agonists (58).

Single-channel analysis reveals that the graded activation of AMPA receptors by 5-substituted willardiines occurs because partial agonists produce preferential activation of lower conductance substates, whereas full agonists preferentially activate higher conductance substates. However, the conductance of the individual substates is the same for full and partial agonists (69). This important result indicates that while the agonist-binding domain can adopt a range of conformations from open to closed, the ion channel likely populates a discrete set of conformations as defined by ion flux. The nature of the coupling mechanism that permits this will likely be understood only when structures of the agonist-binding core attached to the ion channel are solved. This remains a formidable challenge.
Figure 6  Correlation between agonist efficacy and GluR2 S1S2 linker separation. Agonist efficacies were measured from nondesensitizing GluR2-L483Y receptors at a saturating concentration of agonist (58, 66, 69). Efficacies are scaled relative to glutamate which is arbitrarily set to 1. The APO, DNQX, and ATPO complexes are assumed to have an efficacy of zero. Linker separation is defined as the distance between the Cα atoms from the glycine residue in the engineered Gly-Thr S1S2 linker. The corresponding crystal structure is indicated next to each point. IW linker separation was also calculated as described by Jin et al. (69) and is indicated by a square. The linear fit to the agonist-bound points (excluding IW) yields a correlation coefficient of 0.921.

AMPA RECEPTOR DESENSITIZATION AND THE DIMER INTERFACE

A striking feature of iGluR responses, particularly for AMPA and kainate receptors, is the rapidity of onset and extent of desensitization in response to the sustained application of agonist. From the beginning of studies on iGluRs, it was recognized that desensitization resulted from a conformational change intrinsic to
the receptor itself, but the nature of this process remained obscure for more than a decade. The first clues came from mapping the results from functional studies onto the GluR2 agonist-binding core crystal structure. This revealed that amino acid substitutions in the flip-flop splice variants of AMPA receptors, which regulate sensitivity to allosteric modulators of desensitization (70), lie on the dimer interface of the agonist-binding core. Likewise, the L483Y mutant, which attenuated the desensitization of GluR3 (71), was also found to lie on the dimer interface.

Establishment of the mechanism by which allosteric modulators and point mutants in the dimer interface regulate the process of desensitization was obtained from a combination of biochemical and electrophysiological approaches. Using analytical ultracentrifugation to measure the dissociation constant for dimer formation by the isolated GluR2 agonist-binding core, Sun et al. found that mutations that attenuate desensitization result in a more stable dimer complex, whereas mutations that disrupt the dimer interface accelerate the rate of onset and increase the extent of desensitization (72). A striking correlation between the extent of equilibrium desensitization in full-length receptors and the dissociation constant for dimer dissociation suggests that the processes are causally related. A simple extension of the model proposed for activation can accommodate the process of desensitization if it is assumed that the agonist binding energy is available either to open the ion channel gate or to rearrange the dimer interface, allowing the protein to relax into a low-energy state in which the agonist-binding cores remain in their active, closed cleft conformation while the ion channel closes (Figure 3). In retrospect it is clear that activation and desensitization are linked processes that use a common structural element, namely the dimer interface.

Consistent with the above picture was the discovery that cyclothiazide, an allosteric modulator of desensitization, stabilized the formation of dimers by the isolated GluR2 agonist-binding core. The crystal structure of a ternary complex of cyclothiazide, AMPA, and GluR2 S1S2 revealed that two cyclothiazide molecules were bound at the base of the dimer interface, making hydrogen bond contacts with the alternatively spliced serine side chain of the cyclothiazide-preferring flip splice variant at the base of helix J and gluing the subunits together (72).

**STRUCTURAL STUDIES ON NMDA RECEPTORS**

For reasons that are not yet clear, some iGluR S1S2 constructs express well and can be crystallized without difficulty, whereas others have resisted attempts at protein expression. Fortunately, the NR1 glycine-binding site of the NMDA receptor gene family has been expressed and crystallized in complex with a series of agonists and antagonists (50). As expected, glycine and other NR1-preferring ligands bind in a cleft between the two lobes of the S1S2 construct, comparable to the mechanism observed for periplasmic proteins, GluR0, GluR2, and GluR6. The fold of NR1 shows two notable differences from that of GluR2 and GluR6. First, the lengths of loop one and loop two are increased, compared with those in AMPA and kainate
receptors, and in combination form a prominent ridge on the external surface of domain 1, which might be expected to form an interaction surface for contact with another subunit, or perhaps the N-terminal domain (Figure 1). Second, the orientation of one of the domain 2 helices (G) differs substantially from that in GluR2 and GluR6. Despite this, AMPA, kainate, and NMDA receptors share many common features in their ligand-binding sites.

The structure of the NR1 S1S2 agonist-binding core explains clearly why glycine is bound with high affinity, how other much larger agonists and partial agonists fit into the ligand binding pocket, and why glutamate is excluded. Despite its simple chemistry, glycine makes a total of eight hydrogen bonds and ion pair interactions with the NR1 agonist-binding site. In addition, five structural water molecules are trapped together with glycine in the closed cleft conformation. The glycine-water complex makes a network of hydrogen bond contacts that stabilizes the agonist-bound closed cleft conformation. S-glutamate is prevented from binding by a steric barrier formed by the replacement of a leucine in GluR2 by Trp731 in NR1, the large aromatic side chain of which would collide with the \( \gamma \)-carboxyl group of glutamate. In addition, there is loss of a hydrogen bond donor at the base of helix F, which occurs on replacement of a threonine in GluR2 by Val689 in NR1. In combination, these amino acid substitutions make the binding of glutamate to NR1 too weak to detect.

Similar to GluR2 S1S2 complexes with competitive antagonists, NR1 adopts an open cleft conformation when crystallized with the antagonist, 5,7-dichlorokynurenic acid (DCKA). DCKA acts as a wedge, or foot-in-the-door, preventing closure of the NR1 agonist-binding core. The binding of the planar bicyclic DCKA antagonist molecule is stabilized in part by stacking interactions with aromatic amino acids, reminiscent of that seen for the GluR2 complex with DNQX. Comparison of the DCKA and glycine-bound complexes reveals a 24° difference between the open and closed cleft conformations, 3° larger than that observed for GluR2 complexes. Of note, the two protomers in the asymmetric unit of the NR1 DCKA complex differ in the extent of domain closure by 6°; at present the structure of an apo complex has not been reported, but it is likely that, as observed for GluR2 DNQX and ATP complexes, NMDA receptor glycine site antagonists can also produce moderate domain closure.

Although several lines of evidence support the conclusion that functional NMDA receptors assemble as heteromers of two NR1 and two NR2 subunits (73–75), it remains unclear how the subunits are arranged around the ion channel pore and whether they form homo- or hetero-dimers. The NR1 S1S2 construct used by Furukawa & Gouaux is monomeric in solution, and a dimerization interface similar to that observed for GluR2 and GluR0 is absent from the crystal lattice (50). The implications of this result are unclear as the literature contains conflicting reports on the association between NMDA receptor subunits. The N-terminal domain of NR1 is required for homodimerization of soluble NR1 constructs (22), and an NR1 S1S2 construct slightly longer than the one crystallized by Furukawa & Gouaux also forms dimers in solution (47). Together these results suggest that NR1 does
form homodimers but that the dimerization energy was too high to compete with lattice contacts elsewhere in the crystal. Recent work using tandem-linked NMDA receptor subunits suggests a 1-1-2-2 arrangement as opposed to a positioning of subunits kitty-corner to each other (1-2-1-2) (41).

FUNCTIONAL STUDIES OF IGLURS IN THE CRYSTALLIZED STATE

An issue that frequently arises during crystallographic studies of isolated protein domains is how to relate these structures to the function of the intact receptor. There is also the valid concern that, when packed in a crystal lattice, the conformations observed may not accurately reflect those of the protein in solution. While this must always be addressed on a case-by-case basis, some elegant experiments suggest that in the case of GluR2 the behavior of the agonist-binding core in the crystal is remarkably close to that observed in solution (65). Crystal-soaking experiments reveal that GluR2 S1S2 AMPA complexes have sufficient conformational mobility to allow AMPA to unbind and be displaced by the partial agonist 5-Br-willardiine, which then stabilizes the agonist-binding core in the partially closed conformation characteristic of partial agonists. The experiments that established this used the anomalous scattering of X rays by bromine atoms to measure the binding of 5-Br-willardiine in the presence of AMPA. Crystals of the GluR2 S1S2 AMPA complex were washed in a solution containing 350 nM AMPA and then soaked in a solution containing 350 nM AMPA and various concentrations of 5-Br-willardiine. An EC\textsubscript{50} of 12.9 µM was determined for development of an anomalous signal owing to binding of 5-Br-willardiine that displaced AMPA from crystallized iGluR2. In independent experiments with soluble GluR2 S1S2, an IC\textsubscript{50} of 4.0 µM was measured for displacement of 350 nM [\textsuperscript{3}H]-AMPA by 5-Br-willardiine. Measurements of the decrease in domain closure that occurred during the crystal titration experiment gave an EC\textsubscript{50} of 6.8 µM. In total, these experiments suggest that despite the constraints imposed by the crystal lattice, GluR2 S1S2 molecules have sufficient mobility to allow agonists to bind and dissociate and to undergo global conformational changes induced by the binding of full and partial agonists.

CONCLUSION

The physiological role of iGluRs is now well established, but even this maturing field remains an active area of research because of the continuing discovery of novel regulatory mechanisms. By comparison, analysis of the molecular basis of iGluR function is an emerging research area, and even though the knowledge gained from the studies reviewed here is without precedent, major questions remain to be answered. Determining the structure of a full-length vertebrate iGluR in its
native oligomeric state will probably remain an elusive goal for some time. This is because techniques for the overexpression and purification of eukaryotic ion channel proteins are in their infancy. However, such structures will ultimately be required to fully understand the processes of activation, desensitization, allosteric modulation, and protein-protein interactions with other components of the synaptic membrane.

Expression of prokaryotic ion channel genes in E. coli has proven to be significantly more tenable than expression of eukaryotic genes. Fortunately for ion channel structural biologists, the rapidly increasing number of bacterial genome sequences has revealed that these organisms harbor homologues to virtually every family of eukaryotic ion channel. Several of these genes have been successfully overexpressed in E. coli, extracted from cell membrane with detergents, purified and crystallized. As proof that these homologues are likely to provide the route to a crystal structure of an intact glutamate receptor, at present every ion channel crystal structure in the Protein Data Bank is derived from a prokaryotic organism, with the exception of the nicotinic acetylcholine receptor (78). Thus far GluR homologues have been identified in at least nine bacterial genome sequences. This growing family of bacterial glutamate receptor ion channels, especially those for which sequence analysis predicts permeability to both Na\(^+\) and K\(^+\), are interesting and promising crystallographic targets. However, because the currently known bacterial iGluRs lack the N-terminal domain and third TM segment of their vertebrate counterparts, there are likely to be significant differences in their structure and function.

Stepping backward from the challenging goal of an intact receptor assembly, solving the structures of the N-terminal domain, and especially the N-terminal domain S1S2 complex, which makes up the whole of the extracellular portion of iGluRs, would also be a major advance. The acetylcholine-binding peptide represents an analogous case for nicotinic and related GABA, glycine, and 5-HT3 receptors. Crystallization of this protein revealed for the first time the structure of the pentameric assembly that forms the ion channel vestibule and agonist-binding site (76). In the case of iGluRs, a comparable structure would be expected to reveal the packing of the N-terminal domain and S1S2 domains, as well as the symmetry of an iGluR tetramer that currently is believed to involve assembly of the ligand-binding core as a dimer of dimers. Although this involves a symmetry violation on moving from the twofold axis of the ligand-binding core dimers to the fourfold axis of the ion channel (72), such an arrangement apparently occurs in the inward rectifier K\(^+\) channels (77).

An immediately accessible, and ongoing research area is the determination of structures for the ligand-binding cores of additional members of the iGluR gene family; also useful would be the structures of complexes with additional agonists and antagonists because this would provide the information required for design and synthesis of novel iGluR subtype–selective ligands. The availability of such compounds would greatly advance our understanding of synaptic physiology and the complex functional roles that iGluRs play in CNS function.
The study of glutamate receptor function has advanced with unprecedented speed during the past few years, and no other neurotransmitter receptor family is as well characterized in terms of structure and mechanism, although it could be argued that nicotinic acetylcholine receptors come close (78). It is hoped that this rate of progress will continue and extend to other receptors and ligand-gated channels as well as to the complex of molecules involved in synaptic transmission.

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LITERATURE CITED

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Figure 1  Domain organization of eukaryotic and prokaryotic iGluRs. The S1 peptide segment that precedes the two-TM pore loop insertion is colored cyan; the S2 peptide segment is colored pink. The S1 and S2 segments cross over into domain 2 and domain 1, respectively. Agonists and competitive antagonists bind in a fissure located between domain 1 and 2. Eukaryotic iGluRs have additional structural elements consisting of an N-terminal domain, a third TM segment, and a cytoplasmic domain. The S1S2-ligand binding core was expressed as a soluble protein by genetically excising the ion channel pore, N-terminal domain, and the C-terminal domain as required.
Figure 2  Crystal structures of the ligand-binding cores of (a) GluR0, (b) GluR2, (c) GluR6, and (d) NR1. The ribbon diagrams are colored using the scheme from Figure 1 with S1 colored cyan and S2 colored pink. Domain 1 is at the top and domain 2 at the bottom. Loops that are present in GluR2, GluR6, and NR1, but not in GluR0, are colored yellow. The disulfide bond that links the C terminus of S2 to domain 1 is drawn in green; in GluR6 the C terminus was disordered, and there was no electron density after the end of the last helix in domain 2. The ball and stick models show glutamate (or, for NR1, glycine) bound in the cleft between domains 1 and 2. Note the different conformation for the glutamate molecule in GluR0 compared with that in GluR2 and GluR6.
Figure 3  State diagram for activation and desensitization of glutamate receptor ion channels. Shown schematically are a pair of ligand-binding cores and their associated ion channel segments. Proceeding from the resting state, the binding of glutamate is shown by attachment of a red ball to domain 1; this triggers domain closure and ion channel activation (*top row*). The bottom row shows entry into desensitized states that results from relaxation of the dimer interface into a conformation that permits both the agonist-binding core and the ion channel to remain in closed conformations. Shown on the bottom left is a hypothetical reaction scheme for activation and desensitization with the coordinate for wild-type plotted in black. Mutations that increase the stability of the dimeric assembly (*green*) deepen the energy well for the open state of the ion channel (A2O2) and raise the barrier for entry into the desensitized state (A2D2). Mutations that destabilize the dimer interface (*red*) have the opposite effect.
Figure 4  (A) Amino acid sequence alignments for the pore loop and inner helix of K⁺ channels and iGluRs. Conserved residues in the ion-binding site are colored orange; glycine residues are shaded in red. The location of the gating hinge in MthK is indicated by an arrow. A dashed line separates K⁺-selective prokaryotic iGluRs from those that lack the K⁺ channel signature sequence and would be expected to be permeable to both Na⁺ and K⁺. (B) Amino acid sequence alignments of the TM segments of eukaryotic iGluRs. Conserved glycine residues are shaded in red. The location of glycine residues varies between subtypes.
Figure 5  Agonist subsite diagram for selected GluR2 S1S2 crystal structures. Each GluR2 S1S2 agonist complex was superimposed, using only domain 1 residues, on the S1S2 glutamate structure. The \(\alpha\)-carboxyl and \(\alpha\)-amino groups occupy subsites A and B, respectively. Subsite C is an aliphatic region capped by Tyr450, which is not visible in the orientation shown here (53). The \(\gamma\)-anionic moieties occupy subsites D, E, and F. Subsite G is a large hydrophobic pocket in domain 1, which is occupied by the 5-substituents from AMPA, ACPA, ATPA, and 2-Me-Tet-AMPA. The bottom row contains the diagrams for the partial agonists, kainate, Br-HIBO, and 5-Br-willardine. Notice that all three partial agonists have substituents located outside the subsite map. Oxygen atoms are red, nitrogen atoms are blue, bromine atoms are purple, and water molecules are green. Subsites A, B, and G are formed by residues from domain 1 and are colored blue; subsites D, E, and F are formed by residues from domain 2 and are colored pink.
CONTENTS

Frontispiece—S. Jonathan Singer  xiv

PERSPECTIVES, Joseph F. Hoffman, Editor
Some Early History of Membrane Molecular Biology,  
S. Jonathan Singer  1

CARDIOVASCULAR PHYSIOLOGY, Jeffrey Robbins, Section Editor
Myocardial Aging and Senescence: Where Have the Stem Cells Gone?,  
Mark A. Sussman and Piero Anversa  29
Viral-Based Myocardial Gene Therapy Approaches to Alter  
Cardiac Function, Matthew L. Williams and Walter J. Koch  49

CELL PHYSIOLOGY, Paul De Weer, Section Editor
Developmental Regulation of Lung Liquid Transport, Richard E. Olver,  
Dafydd V. Walters, and Stuart M. Wilson  77
Mechanism of Rectification in Inward-Rectifier K+ Channels, Zhe Lu  103
Metabolic Regulation of Potassium Channels, Xiang Dong Tang,  
Lindsey Ciali Santarelli, Stefan H. Heinemann,  
and Toshinori Hoshi  131
Structure and Function of Glutamate Receptor Ion Channels,  
Mark L. Mayer and Neali Armstrong  161

COMPARATIVE PHYSIOLOGY, George N. Somero, Section Editor
Biochemical Indicators of Stress and Metabolism: Applications  
for Marine Ecological Studies, Elizabeth P. Dahlhoff  183
Field Physiology: Physiological Insights from Animals in Nature,  
Daniel P. Costa and Barry Sinervo  209
Metabolic Rate and Body Temperature Reduction  
During Hibernation and Daily Torpor, Fritz Geiser  239
Sleep and Circadian Rhythms in Mammalian Torpor, H. Craig Heller  
and Norman F. Ruby  275

vii
CONTENTS

ENDOCRINOLOGY, Bert W. O'Malley, Section Editor

Estrogens in the Nervous System: Mechanisms and Nonreproductive Functions, Adriana Maggi, Paolo Ciana, Silvia Belcredito, and Elisabetta Vegeto 291

The Role of Corepressors in Transcriptional Regulation by Nuclear Hormone Receptors, Martin L. Privalsky 315

GASTROINTESTINAL PHYSIOLOGY, John Williams, Section Editor

Molecular and Integrative Physiology of Intestinal Peptide Transport, Hannelore Daniel 361

Oral Rehydration Therapy: New Explanations for an Old Remedy, Mrinalini C. Rao 385

Recent Advances in Carrier-Mediated Intestinal Absorption of Water-Soluble Vitamins, Hamid M. Said 419

NEUROPHYSIOLOGY, Richard Adrich, Section Editor

Learning Mechanisms in Addiction: Synaptic Plasticity in the Ventral Tegmental Area as a Result of Exposure to Drugs of Abuse, Julie A. Kauer 447

Localization of Voltage-Gated Ion Channels in Mammalian Brain, James S. Trimmer and Kenneth J. Rhodes 477

Myosin-1c, the Hair Cell’s Adaptation Motor, Peter G. Gillespie and Janet L. Cyr 521

RENAL AND ELECTROLYTE PHYSIOLOGY, Steven C. Hebert, Section Editor

Regulation of Renal K Transport by Dietary K Intake, WenHui Wang 547

The Extracellular Cyclic AMP-Adenosine Pathway in Renal Physiology, Edwin K. Jackson and Dubey K. Raghvendra 571

RESPIRATORY PHYSIOLOGY, Carole R. Mendelson, Section Editor

Alterations in SP-B and SP-C Expression in Neonatal Lung Disease, Lawrence M. Nogee 601

Epithelial-Mesenchymal Interactions in the Developing Lung, John M. Shannon and Brian A. Hyatt 625

Genetically Engineered Mouse Models for Lung Cancer, I. Kwak, S.Y. Tsai, and F.J. DeMayo 647

SPECIAL TOPIC: PROTON AND ELECTRON TRANSPORTERS, Janos K. Lanyi, Special Topic Editor

Bacteriorhodopsin, Janos K. Lanyi 665

The Cytochrome bc₁ Complex: Function in the Context of Structure, Antony R. Crofts 689
CONTENTS

SPECIAL TOPIC: FUNCTIONAL IMAGING IN PHYSIOLOGY,
   Stephen J Smith, Special Topic Editor
   Interpreting the BOLD Signal, Nikos K. Logothetis and Brian A. Wandell 735
   Live Optical Imaging of Nervous System Development, Cristopher M. Niell and Stephen J Smith 771

SPECIAL CHAPTER: MUSCLE PHYSIOLOGY, Joseph F. Hoffman, Editor
   Control of the Size of the Human Muscle Mass, Michael J. Rennie, Henning Wackerhage, Espen E. Spangenberg, and Frank W. Booth 799

INDEXES
   Subject Index 829
   Cumulative Index of Contributing Authors, Volumes 62–66 879
   Cumulative Index of Chapter Titles, Volumes 62–66 882

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