An Amino Acid Substitution in the Pore Region of a Glutamate-gated Chloride Channel Enables the Coupling of Ligand Binding to Channel Gating*

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Many of the subunits of ligand-gated ion channels respond poorly, if at all, when expressed as homomeric channels in Xenopus oocytes. This lack of a ligand response has been thought to result from poor surface expression, poor assembly, or lack of an agonist binding domain. The Caenorhabditis elegans glutamate-gated chloride channel subunit GluClβ responds to glutamate as a homomeric channel while the GluClα subunit is insensitive. A chimera between GluClα and GluClβ was used to suggest that major determinants for glutamate binding are present on the GluClα N terminus. Amino acid substitutions in the presumed pore of GluClα mark-ferred direct glutamate gating indicating that GluClα is deficient in coupling of ligand binding to channel gating. Heteromeric channels of GluClαβ may differ from the prototypic muscle nicotinic acetylcholine receptor in that they have the potential to bind ligand to all of the subunits forming the channel.

Ligand-gated ion channels form a gene superfamily composed of cation channels gated by acetylcholine and serotonin, and anion channels gated by γ-aminobutyric acid (GABA), glycine, and glutamate (1–6). The subunits share a common putative structure of a large hydrophilic extracellular domain thought to contain the ligand binding region and four membrane spanning domains (M1-M4) (2–7). GluClα is a pentamer with the M2 domain of each subunit lining the pore (2–7). GluClα and GluClβ are subunits of a glutamate-gated chloride channel (GluCl) from the nematode Caenorhabditis elegans (1). They are 45% identical on the amino acid level, and closely related to the glycine and GABA receptors (1).

The prototype of a ligand-gated ion channel is the muscle nicotinic acetylcholine receptor where the major determinants of ligand binding are located on the α subunits (2–4). Binding of two agonist molecules leads to channel gating. The number of agonist molecules necessary to gate ligand-gated chloride channels, as well as the subunits which bind ligand remain matters of controversy (5, 6). GABA receptors have an unknown stoichiometry and there is evidence for ligand binding determinants on α, β, γ, and perhaps δ subunits. Glycine receptors are thought to have a stoichiometry of 3α2β2, with the α subunits carrying the ligand binding domains (5). However, very small responses to glycine in Xenopus oocytes injected with the glycine β subunit suggest that a glycine binding site exists on the β subunit (8).

When co-expressed in oocytes, GluClα and GluClβ form heteromeric channels gated by glutamate and the widely used antiparasitic agent ivermectin (1). Ivermectin is a widely used broad spectrum anthelminthic/insecticidal agent used to control parasitic nematodes in man and animals (9). Ivermectin potentiates the glutamate response of heteromeric GluClαβ channels indicating that ivermectin acts as an allosteric modulator of the channel (1, 10). Homomeric GluClβ channels are directly gated with glutamate demonstrating that GluClβ contains all the determinants for ligand binding and coupling to channel gating (1). Homomeric GluClα channels are insensitive to glutamate, but are directly activated by ivermectin (1). We demonstrate that GluClα is a ligand binding subunit deficient in coupling to channel gating. Heteromeric channels of GluClαβ have the potential to bind ligand to all of the subunits forming the channel.

EXPERIMENTAL PROCEDURES

Electrophysiological Recordings—Xenopus oocytes were prepared, injected (in vitro RNA, 0.1–10 ng), and membrane currents recorded as described previously (10, 11). Recordings were made in standard frog saline consisting of (in mM): NaCl (115), KCl (2), MgCl2 (1), CaCl2 (1.8), HEPES (10) adjusted to pH 7.5 with NaOH.agarase-cushion electrodes were made as described (12). The water soluble derivative 22,23-dihydroivermectin B1α, 4'-O-phosphate (IVMPO4) was used for these studies (11). Concentration-response curves were fit to a modified Michaelis–Menten equation:

\[ I_{\text{max}} = \frac{I_{\text{max}}}{1 + (D/C_{\text{EC50}})^h} \]

where \( I_{\text{max}} \) is the maximal response, \( D \) is the drug concentration, \( C_{\text{EC50}} \) is the drug concentration for half-maximal effect, and \( h \) is the Hill coefficient. All data in text and figures are means ± S.E.

Current voltage (I/V) relationships were determined using 1-s voltage ramps from ~100 to 60 mV in the presence and absence of IVMPO4. Drug-sensitive currents were generated by subtracting drug-free from drug-containing data. Ion substitutions were accomplished by replacing NaCl in standard frog saline with KCl, cholineCl, or sodium isethionate as indicated. The bath was connected to the ground through a 3M KCl-agar bridge for experiments in which external chloride was replaced. To limit contributions from calcium-activated chloride currents to the I/V relationships, CaCl2 was removed from the external solutions, and oocytes were preincubated containing 10 μM BAPTA-AM (13).

Design and Construction of Molecular Clones—Homologous positions between ligand-gated ion channel subunits were determined using the pretty plot alignment from the GCG package, version 7 (Genetic Computer Group, Madison, WI). The EcoRI restriction sites were introduced by methods described in Ref. 14. The chimeric receptor was constructed as a Sall-EcoRI fragment of GluClα and a EcoRI-NotI fragment of GluClβ. Base pair substitutions in the codon for amino acid 308 in...
channels between the 5HT1C and a7 nicotinic acetylcholine receptor further confirmed this localization (18). To determine if the extracellular domain of GluClα contains major components of a glutamate binding site we constructed a chimera between GluClα and GluClβ (GluClα-N-βC) (Fig. 2A). A glutamate-sensitive current with an EC_{50} of 530 ± 90 nM was observed when oocytes were injected with RNA from GluClα-N-βC (Fig. 2B). Ibotenic acid (500 nM) activated 82 ± 3% (n = 3) of the current activated with 500 nM glutamate. No glutamate, GABA, and glycine (1 μM) were inactive. This pharmacology is consistent with the agonist profile of glutamate-gated chloride channels and the IVMPO_{4-dependent} glutamate current of GluClα. Oocytes injected with the chimera also had a large holding current and a resting potential close to the Nernst potential for chloride (−31 ± 2 mV, n = 10). The holding current was inhibited to the level of a water-injected oocyte with 50 μM picrotoxin (n = 7, Fig. 2C), a non-competitive inhibitor of ligand-gated anion channels (10, 17, 19, 20). The current/voltage relationship of the leak current was strongly outwardly rectifying, similar to the rectification of the GluClβ pore (1). The results with GluClα-N-βC suggest that the N-terminal extracellular domain of GluClα contains major determinants of glutamate binding. They also suggest that the C-terminal part of GluClα does not couple ligand binding to channel gating.

Point Mutations in M2 Creates a Glutamate-sensitive Channel—Amino acid substitutions in the C-terminal part of the GluClα channel showed that it was possible to obtain direct glutamate gating in the absence of an IVMPO_{4-induced} conformational change. The amino acid threonine in position 308 was replaced with amino acids known to occur in the homologous position of ligand-gated ion channels (Ala, Gly, Ser, Cys, Pro, Val, and Leu) (Fig. 3A). The mutant channels were assayed for expression by using 5 μM IVMPO_{4}. All mutant subunits except T308L expressed robust (≥1 μA) IVMPO_{4}-sensitive currents. The EC_{50} for IVMPO_{4} was not dramatically altered in the mutants as judged by the threshold concentration of IVMPO_{4} needed to activate a current (50–100 nM, data not shown). No glutamate-induced currents (10 and 50 mM) were observed for T308S, T308C, T308V, T308L. However, the mutants T308A, T308P with an EC_{50} of 1.4 mM followed by T308A and T308G showed an increased rate of desensitization in the presence of IVMPO_{4}. This phosphoesterase of glutamate-gated chloride channels (1–10 mM) was inactive. These results demonstrate that homomeric GluClα channels contain, in the absence of IVMPO_{4}, all the determinants for a glutamate binding site.

The amino acid substitutions at position 308 of GluClα also altered the rate of desensitization of the glutamate- and IVMPO_{4}-sensitive currents (Fig. 3, B and C). This result is consistent with the alterations in desensitization properties associated with this position in a Drosophila GABA receptor (21). The mutations T308G, T308A, T308C, and T308P all responded to glutamate (Fig. 3A). The amino acid substitutions at position 308 of GluClα were the following: GCC (glycine), GTC (valine), AGC (serine), TGC (cysteine), CCC (proline), CTC (leucine), and GCC (alanine). Constructs were cloned into a pBluescript II KS vector and both strands were sequenced. Constructs were linearized with NotI or SacI and in vitro transcribed RNA was performed as described (1).

RESULTS
IVMPO_{4} Reveals a Cryptic Glutamate Binding Site—Homomeric GluClα channels are not directly gated by glutamate over a concentration range of 0.01–50 mM (Fig. 1A) (1). Surface expression of functional homomeric GluClα channels was revealed with IVMPO_{4}, which irreversibly activated current (Fig. 1A) (1). Interestingly, glutamate elicited a small reversible increase in current after activation with IVMPO_{4} (Fig. 1A) (1). The glutamate-sensitive current was only observed if channels were first activated with IVMPO_{4}. On average, 10 μM glutamate increased current an additional 9.3 ± 1.2% (n = 5) after IVMPO_{4}. The IVMPO_{4}-dependent glutamate current had an EC_{50} for glutamate of 7 ± 2 μM, which is an approximately 50-fold increase in sensitivity when compared to homomeric GluClβ channels (Fig. 1B). Ibotenic acid (10 μM), a structural analog of glutamate known to activate glutamate-gated chloride channels (1, 10, 15–17), increased the current by 8.1 ± 1% (n = 5) after 1 μM IVMPO_{4}. Similar to other glutamate-gated chloride channels o-glutamate (10 μM), GABA (1 mM), and glycine (1 mM) were inactive (1, 10, 15–17). These results suggest that GluClα contains a cryptic glutamate binding site.

The Extracellular Domain of GluClα May Contain Determinants of Glutamate Binding—The major determinants of the ligand binding site in ligand-gated ion channels are thought to reside on the large extracellular N-terminal (3–6). Chimeric

![Figure 1](image-url)

**Figure 1.** IVMPO_{4}-dependent glutamate current. A, membrane currents recorded at a holding potential of −80 mV from oocytes injected with GluClα (1 ng). Solid lines above current traces indicate the time for which the perfusion system was switched to drug-containing solution. The time between current traces was 1 min. **B**, concentration dependence of the IVMPO_{4}-dependent glutamate current. Varying concentrations of glutamate were applied after activation of current with 200 nM IVMPO_{4}. Maximal current was activated with 1 mM glutamate. The smooth curve represents the best fit equation with an EC_{50} of 7 μM (h = 3.0, n = 5). The dashed curve represents the concentration relationship for homomeric GluClβ channels (EC_{50} = 380 μM, h = 1.9) (1).
T308P was independent of the alterations in desensitization. Mutations at position 308 had little effect on the pore properties of homomeric channels (Fig. 4). The IVMPO-δ-sensitive current of GluClα (1) and the T308P mutant had linear I/V curves, while the I/V curve of T308A was slightly outwardly rectifying. All were anion selective since replacement of extracellular sodium with potassium or choline had no effect on $E_{\text{rev}}$, or the shape of the current/voltage relationship (data not shown). The reversal potentials after replacement of extracellular NaCl with sodium isethionate were $26\pm 1$ (GluClα), $36\pm 4$ (T308P), and $1\pm 1\, \text{mV}$ (T308A), indicating a permeability ratio of isethionate to chloride of approximately 0.2 (1, 22, 23).

**DISCUSSION**

We present three independent lines of evidence that homomeric GluClα channels contain a glutamate binding site with pharmacology common to glutamate-gated chloride channels. First, we observe an IVMPO-δ-dependent glutamate current indicating that IVMPO-δ induced a conformational change that allowed gating of the channel with glutamate (Fig. 1). Second, we show that the large extracellular loop of GluClα is sufficient to cause the gating of a GluClβ pore (Fig. 2). This result suggests that major determinants of glutamate binding are present on the GluClα N-terminal. Most importantly, we were able to restore the complex mechanism of ligand binding to channel gating in a homomeric GluClα channel by replacing one amino acid in the pore region (Fig. 3). Changing the naturally occurring threonine in M2 to a proline, glycine, or alanine enabled glutamate gating of GluClα homomeric channels. Gating was independent of the side chain volume or the chemical properties of the amino acid. This result suggests that a subtle conformational change, like the introduction of a bend by proline or flexibility by glycine, was sufficient to reduce the energy needed for channel gating. A reduction in the free energy necessary to couple ligand binding to channel gating has also been observed for point mutations in the second membrane spanning domain of nicotinic acetylcholine receptors (24, 25).

GluClα is a clear example of a ligand binding subunit which fails to couple ligand binding to channel gating as a homomeric channel. Other subunits of the ligand-gated ion channel superfamily that express poorly with low apparent affinity as homomeric channels may simply have poor coupling of ligand binding to channel gating.

Our results indicate that two separate subunits in heteromeric GluClα+β channels carry independently all the determinants for ligand binding. It is tempting to speculate that coassembly with GluClβ provokes a subtle conformational change which leads to the coupling of the GluClα binding site to channel gating. The interactions of these binding sites in a hetero-

**Fig. 2. Chimeric receptor.** A, schematic representation of the strategy used to construct the chimera along with the proposed topology for ligand-gated ion channels. Parts of the chimera contributed by GluClα (black) and GlusClβ (gray) are indicated. Amino acids are in single letter code. Boxes represent conserved amino acids in the GluClα and GlusClβ protein. Nucleotides coding for amino acids represented in bold have been modified to include an EcoRI restriction enzyme site. The four predicted membrane spanning domains (M1-M4) are boxed and labeled. B, glutamate dose-response curve of the chimera. The inset shows membrane currents elicited with 0.3, 0.9, and 10 μM glutamate. The smooth curve represents the best fit to Equation 1 with an EC₅₀ of 530 nM (h = 1.2, n = 5). C, inhibition of leak current with picrotoxin.
meric channel remains to be determined. Perhaps, binding to one type of ligand binding site determines channel opening while binding to another type influences desensitization. Alternatively, subconductance states commonly observed in ligand-gated chloride channels (26, 27) may be governed by number and type of ligand binding sites occupied. The scheme of bindingsites on more than one type of subunit probably carries over to other members of the ligand-gated chloride channel family. For example, there is evidence for homomeric channel formation of glycine \( \beta \) subunits (8). Homomeric \( \alpha \) and \( \beta \) subunits of the GABA \( \alpha \) receptor have been expressed, however, with poor efficiency (28, 29). Furthermore, ligand binding determinants for GABA binding may be present on the \( \alpha \), \( \beta \), \( \gamma \), and \( \delta \) subunits (6). Our results suggest that heteromeric GluCl- \( \beta \) channels differ from prototypical muscarinic acetylcholine receptor in that more than one type of subunit carries major determinants for ligand binding.

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