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Unique Contributions of an Arginine Side Chain to Ligand Recognition in a Glutamate-gated Chloride Channel*\textsuperscript{[S]}

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Edited by F. Anne Stephenson

Glutamate recognition by neurotransmitter receptors often relies on Arg residues in the binding site, leading to the assumption that charge-charge interactions underlie ligand recognition. However, assessing the precise chemical contribution of Arg side chains to protein function and pharmacology has proven to be exceedingly difficult in such large and complex proteins. Using the in vivo nonsense suppression approach, we report the first successful incorporation of the isosteric, titratable Arg analog, canavanine, into a neurotransmitter receptor in a living cell, utilizing a glutamate-gated chloride channel from the nematode Haemonchus contortus. Our data unveil a surprisingly small contribution of charge at a conserved arginine side chain previously suggested to form a salt bridge with the ligand, glutamate. Instead, our data show that Arg contributes crucially to ligand sensitivity via a hydrogen bond network, where Arg interacts both with agonist and with a conserved Thr side chain within the receptor. Together, the data provide a new explanation for the reliance of neurotransmitter receptors on Arg side chains and highlight the exceptional capacity of unnatural amino acid incorporation for increasing our understanding of ligand recognition.

Neurotransmitter receptors are vital signaling proteins that are embedded in the cell membrane and trigger intracellular changes in response to extracellular chemical signals. The two classical receptor types are metabotropic, G-protein-coupled receptors (GPCRs) that act over seconds or minutes via intracellular second messengers (1), and ionotropic, ligand-gated ion channels (LGICs)\textsuperscript{2} that mediate ion flux across the membrane on the millisecond timescale (2). The rapid chemo-electric signaling of LGICs is perfectly suited to the nervous system, where activation of sodium channels and chloride channels mediates excitatory and inhibitory signals, respectively (2). The first step in the process of activation is the recognition of a specific ligand, which in the case of the animal nervous system is very often the neurotransmitter glutamate (3).

Glutamate binding to neurotransmitter receptors has been studied in great detail, reflected in X-ray structures of ligand-receptor complexes of both LGICs (4, 5) and GPCRs (6). Perhaps not surprisingly, each complex contains an Arg side chain in close proximity to at least one of the glutamate carboxylates, suggestive of ionic interactions between negatively charged carboxylate and positively charged guanidino groups. Reduced function upon Ala substitution confirms the importance of these Arg residues in glutamate recognition in each receptor subfamily (7–9), but despite this apparent functional evidence for a charge-charge interaction, replacing a large Arg side chain with a much smaller Ala side chain involves more physicochemical changes than merely removing a positive charge. As such, the precise contribution of highly conserved Arg side chains in ligand recognition remains unknown.

Here, we have sought experimental evidence for charge-charge interactions in ligand recognition, focusing on the AVR-14B glutamate-gated chloride channel (GluCl (10)). GluCls are invertebrate-specific members of the pentameric ligand-gated ion channel (pLGIC or “Cys-loop receptor”) family, sharing significant homology with vertebrate GABA and acetylcholine receptors and constituting an important antiparasitic drug target (11). Despite the fact that a Caenorhabditis elegans GluCl was the first eukaryotic pLGIC to be visualized by X-ray crystallography (5), the molecular basis for neurotransmitter recognition in GluCls has received little experimental interrogation, as compared with vertebrate homologs. It has recently been shown, however, that in GluCls, glutamate recognition involves interactions between aromatic residues on the principal face of the binding site with the glutamate amine (7), as well as interactions between Arg residues in the binding site with the glutamate carboxylate groups (12), as illustrated in supplemental Fig. S1. We replaced Arg\textsuperscript{76} in the glutamate binding site with a titratable amino acid, providing us with the unprecedented opportunity to test glutamate sensitivity when an isosteric side chain is present but charged or uncharged. The results indicate only a small role of positive charge and unveil another unique property of Arg side chains that contributes to glutamate sensitivity, namely the ability to form hydrogen bonds both with the agonist and with vicinal receptor side chains.
FIGURE 1. Arg-Lys substitutions drastically reduce glutamate recognition. A, X-ray structure of GLC-1 GluCl (PDB 3RF; gray shading, notional cell membrane). Magnified view shows glutamate binding site and selected amino acid side chains. These include GLC-1 arginine residues 37 and 56, which are labeled Arg and Arg to describe the equivalent residues from the AVR-14B GluCl used in the present study. B, left, example recordings of glutamate (Glu) and ivermectin (IVM, 1 μM) responses at oocytes expressing mutant AVR-14B GluCls (scale bars: x, 5 s; y, 2 μA). Activation by IVM, which binds elsewhere on the receptor, confirms cell surface expression in the absence of Glu-gated currents. Right, mean ± S.E. (n = 4–8) peak current responses to increasing concentrations of Glu, normalized to maximum Glu-gated current (WT) or maximum IVM-gated current (mutants).

Results

To test whether positive charge of Arg residues 76 and 95 is sufficient for glutamate recognition in the glutamate binding site of the AVR-14B GluCl, we replaced these individually with Lys via site-directed mutagenesis and measured glutamate-gated chloride currents with two electrode voltage clamp experiments (Fig. 1, A and B). Given the water-accessible location of these positions in GluCls (13) and the positive charge on Lys side chains in such environments (14), one would expect glutamate sensitivity of mutant receptors to reflect that of WT receptors if positive charge were the main contribution of these side chains to glutamate recognition with two electrode voltage clamp sensing suppression of ArgUAG mRNA by co-injection of Can-ligated tRNA

FIGURE 2. Incorporation of titratable arginine analog, canavanine. A, L-lysine (Lys), L-arginine (Arg), and L-canavanine (Can). B, graphic illustrating nonsense suppression of Arg95UAG mRNA by co-injection of Can-ligated tRNA into Xenopus laevis oocytes (yellow/brown spheres). C, mean peak current amplitude (± S.E.) in response to 10 mM glutamate at oocytes injected with mRNA and tRNA UAG Can Arg(Can) (Can76) (Can76). Scale bars: x, 10 s; y, 100 nA. E, left, glutamate-gated currents at oocytes expressing wild-type or Can76 GluCls continuously perfused at pH 7.0 or 5.8, as indicated (scale bars: x, 30 s; y, 100 nA). Right, mean ± S.E. peak current responses to 30 μM glutamate, normalized to maximum glutamate-gated current (Can76 vs. Can76 Can Arg(Can) (Can76)). Gray arrows illustrate inhibition of 30 μM glutamate-gated current. Can76 pH 7.0 recording in E is repeated from D.

also observed for Can76, these were not significantly greater than controls lacking Can (Fig. 2C; 18 ± 5 nA, n = 10; 4 ± 2 nA, n = 8). This made further characterization of Can76 receptors difficult, and we chose not to investigate these further.

When we measured glutamate sensitivity of Can76 receptors at pH 7.0, at which one would expect only ∼50% of Can76 side chains to be protonated, we were surprised to find that the EC50 for activation by glutamate was 48 ± 7 μM (n = 6), and thus not

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TABLE 1
Glutamate sensitivity of Can76 and Arg76 GluCls

<table>
<thead>
<tr>
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<th>EC50 (\mu M)</th>
<th>Imax nA</th>
<th>n</th>
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<tr>
<td>Can76</td>
<td></td>
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<tr>
<td>pH 7.0</td>
<td>48 ± 7</td>
<td>60 ± 40</td>
<td>6</td>
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<tr>
<td>pH 8.5</td>
<td>119 ± 11**</td>
<td>214 ± 91</td>
<td>5</td>
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<tr>
<td>pH 9.2</td>
<td>118 ± 12**</td>
<td>110 ± 27</td>
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Arg76 (WT)

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<tbody>
<tr>
<td>pH 7.0</td>
<td>41 ± 11</td>
<td>241 ± 51</td>
<td>8</td>
</tr>
<tr>
<td>pH 8.5</td>
<td>43 ± 5</td>
<td>170 ± 47</td>
<td>8</td>
</tr>
<tr>
<td>pH 9.2</td>
<td>57 ± 7</td>
<td>428 ± 91</td>
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** EC50 value was calculated by plotting current amplitude against glutamate concentration and fitting with Hill equation for experiments at individual oocytes and then averaged. Data are mean ± S.E.

significantly different from WT receptors (41 ± 11 μM, n = 8). At pH 8.5, when even fewer, if any, Can76 side chains are expected to be protonated, we observed only a modest decrease in glutamate sensitivity (Fig. 2D), with the EC50 significantly increased to 119 ± 11 μM (Table 1). A further increase in pH to 9.2 saw no additional rise in EC50 value (Fig. 2D; Table 1), suggesting that the effect was saturated around pH 8.5. No such pH-dependent shift was seen for WT receptors (Table 1), in which Arg76 residues are always protonated. This indicates that a 2-fold decrease in glutamate sensitivity in conditions that deprotonate the Can (not Arg) side chains is specific for receptors incorporating a Can residue in the glutamate binding site. Unfortunately, we could not measure the effects of fully protonated Can76 side chains, as acidic pH causes significant inhibition of function even in WT receptors (Fig. 2E), as is the case in structurally related GABA and glycine receptors (17, 18). To verify the 2-fold decrease in glutamate sensitivity observed with an uncharged analog at position 76, we attempted to replace Arg76 with citrulline, an analog in which one NH nitrogen is replaced by an oxygen and which is uncharged (19), via nonsense suppression, but this was not successful (data not shown). Thus, and although a complete titration could not be completed, our data show that in conditions in which theoretically only 50% of Can76 side chains carry a positive charge, Can76 receptors show very similar glutamate sensitivity to WT Arg76 receptors (Table 1). Perhaps more strikingly, upon deprotonation and loss of positive charge in Can76 receptors, a mere 2-fold reduction in glutamate sensitivity is observed. This is a modest reduction in agonist sensitivity as compared with the 10,000-fold reduction caused by R76N or even R76K mutations in this very receptor (12) (Fig. 1), raising the possibility that a more substantial contribution to glutamate binding derives from some property of the Arg (or, indeed, Can) side chain other than positive charge.

Although some of that contribution is presumably via H-bonds between Arg (or Can) NH2 group(s) and glutamate (Fig. 3A), we considered that the Arg εNH group could also be important. Indeed, we noticed in the GLC-1 GluCl-glutamate X-ray structure (Protein Data Bank (PDB) 3RIF (5)) that although not in direct contact with the bound agonist, the hydroxyl oxygen atom of a Thr residue (equivalent to Thr23 in the AVR-14B GluCl) is located in close (2.9 Å) proximity to the εNH of the Arg equivalent to Arg76 (Fig. 3A). If this potential H-bond were important for glutamate sensitivity, we reasoned that the T93S substitution should retain glutamate sensitivity, as Ser also possesses a β-hydroxyl group. In contrast, Val is sterically similar to Thr but devoid of the hydroxyl, and mutant T93V receptors would be expected to show decreased glutamate sensitivity. Indeed, when we measured glutamate-gated currents at these mutants, T93V receptors showed drastically reduced glutamate sensitivity, barely responding to millimolar concentrations (Fig. 3, B and C). By contrast, T93S receptors showed a 370-fold increase in glutamate sensitivity as compared with WT (Fig. 3, B and C; Table 2), confirming that the hydroxyl at this position is required for regular (or increased) glutamate sensitivity, likely through an interaction with Arg76.

Seeking specific evidence for this interaction, we performed double mutant cycle analysis, according to which the T93S substitution should not restore high glutamate sensitivity on R76K mutant receptors because these WT residues are coupled and the effects of their mutation are not simply additive (20–22). In contrast, the combined effects of mutating independent residues are additive, and the T93S mutation might be expected to confer higher glutamate sensitivity on R95K receptors, as we do not expect coupling between these two residues. Indeed, double mutant T93S/R95K receptors responded to glutamate in a concentration range between T93S and R95K single mutants (Fig.
TABLE 2
Glutamate sensitivity of WT and conventional mutants

<p>| Oocytes were injected with 10 ng of WT or mutant mRNA. *** p &lt; 0.001 as compared with WT (ANOVA with Tukey’s post test). |
|-----------------|-----------------|-----------------|-----------------|</p>
<table>
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<tr>
<th><strong>EC</strong>&lt;sub&gt;G&lt;/sub&gt;</th>
<th><strong>I&lt;sub&gt;max&lt;/sub&gt;</strong></th>
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<tr>
<td>WT</td>
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<tr>
<td>R76K</td>
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<td>T93S</td>
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<td>R95K</td>
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<td>R76K/T93S</td>
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<td>R95K/T93S</td>
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**EC**<sub>G</sub> value was calculated by plotting current amplitude against glutamate concentration and fitting with Hill equation for experiments at individual oocytes and then averaged. Data are mean ± S.E. For certain mutants, saturation in the concentration-response relationship was not reached, up to 30 μM glutamate. EC<sub>G</sub> values are therefore estimated to be well over 100 μM.

Discussion

Taken together, these results suggest that the positive charge of Arg<sup>76</sup> contributes little to glutamate binding in GluCls. Instead, our results show that two other aspects of the Arg side chain contribute to effective glutamate recognition. First, the data suggest that H-bonds between the Arg εNH<sub>2</sub> group(s) and the agonist α-carboxylate are important, as conventional Asn and Lys mutations that remove this moiety of Arg are severely detrimental to glutamate recognition, whereas the non-canonical substitution of Can, which retains εNH<sub>2</sub> groups, retains WT-like glutamate sensitivity. Second, the εNH of Arg appears to interact closely with a vicinal receptor hydroxyl side chain, the removal of which via Val substitution drastically reduces glutamate sensitivity. Based on available GluCl structures, this interaction is likely to stabilize Arg for its interaction(s) with the glutamate α-carboxylate (Fig. 3A). Notably, this Loop D hydroxyl side chain is highly conserved in GluCls that possess the Loop G Arg (Arg<sup>76</sup> in AVR-14B), which interacts with the glutamate α-carboxylate. By contrast, the Loop D hydroxyl is absent in GluCls where instead a Loop A Arg (on the opposing face of the binding site, equivalent to Q141 in AVR-14B) interacts with the glutamate α-carboxylate (Fig. 3A) (12).

Conventional mutagenesis is an indispensable tool for dissection of protein structure and function, but in fine-tuning the details of ligand recognition, it is limited by the numerous physico-chemical changes involved in most substitutions (23). This is perhaps especially the case for Arg, where conventional analogs Lys and His are noticeably smaller and more frequently than Arg uncharged in physiological settings (14, 24). Previous attempts to circumvent the limitations of conventional mutagenesis by incorporating unnatural Arg analogs have been few (19, 25, 26) and arguably difficult (27). Our use of the *in vivo* nonsense suppression method was successful for one of the two positions tested, and despite limited efficiency (currents through Can<sup>76</sup> were substantially smaller than conventional mutant receptors; compare I<sub>max</sub> in Tables 1 and 2), the data strongly support the notion of robust and specific Can incorporation in three ways. First, the level of nonspecific incorporation of endogenous amino acids, as inferred from the very low current levels upon co-injection of uncharged tRNA, was very low (Fig. 2C). Second, although all conventional replacements at position 76 tested here and elsewhere (7, 12) resulted in drastic losses in glutamate sensitivity, we found Can incorporation to yield WT-like glutamate sensitivity (at pH 7.0; Table 1). Lastly, and perhaps most significantly, we found the agonist sensitivity of Can<sup>76</sup> receptors to be titratable between pH 7.0 and 8.5, a property that would be highly unlikely in the event of endogenous amino acids incorporating into this position. We note here that in our hands the incorporation of Can was more successful than citrulline at positions 76 and 95. However, we cannot assess with certainty the reason for this difference, which could be due to ribosomal recognition of the charged tRNA, protein folding, or protein function.

In conclusion, Can incorporation, together with subsequent conventional mutagenesis, has unveiled crucial determinants of ligand recognition that had previously escaped identification. Our results suggest that the common occurrence of Arg residues in glutamate binding sites is related to the ability of Arg side chains to participate in H-bonds both with ligand and with vicinal receptor side chains simultaneously. Thus, the unique propensity of Arg for forming multiple H-bonds, as described previously in the context of intramolecular interactions in other protein families (28), seems to have been utilized by Glu-
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Cys for the specific functional requirements of ligand recognition. The results also complement work on tetrameric ionotropic glutamate receptors (iGluRs), regarding both the unique role of Arg and the occurrence of vicinal hydroxyl side chains that could stabilize binding site architecture. In NMDA-type iGluRs, for example, Lys substitution of the Arg residue that binds the ligand α-carboxylate abolishes glutamate sensitivity (29, 30), much like R76K and R95K substitutions in the GluCl. Remarkably, NMDA-type iGluRs also contain Ser and Thr residues, whose side chain hydroxyl oxygens are, similar to Thr93 in the GluCl, as close as 2.7 Å to other side chains that form the glutamate binding site (29, 31), and whose substitution for Ala drastically reduces glutamate sensitivity (29).

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Experimental Procedures

Chemical Synthesis—All reagents were of analytical grade and were used directly as received. The reagents were purchased from Sigma-Aldrich, unless stated otherwise. LC-MS analyses of synthesized compounds were performed on a Waters ACQUITY ultra high performance liquid chromatography system. Eluents A (0.1% HCOOH in water) and B (0.1% HCOOH in acetonitrile) were used in a linear gradient (100% A to 95% B) in a run time of 2.5 min. HCOOH in acetonitrile (v/v)) were used in a linear gradient (100% A to 95% B) in a run time of 20 min and concentration of ligand binding by such H-bond networks could be a conserved feature of ligand recognition by pLGICs.

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rated with forces and then treated with 0.5 mg/ml Type I collagenase (Worthington Biochemical) in OR2 (in mM, 2.5 NaCl, 2 KCl, 1 MgCl2, 5 HEPES, pH 7.4 with NaOH) with continuous shaking at 200 rpm and 37 °C. These were incubated in OR2 at 18 °C until injection of mRNA. For conventional mutants and WT mRNAs, 10 ng, in a volume of 40 nl (diluted in water), was injected using a Nanoliter 2010 injector (World Precision Instruments). For Can incorporation, 40 ng of UAG-mutant mRNA together with aminoacyl tRNA, in a volume of 40 nl, was injected. For lower expression of WT (Table 1), 0.04 ng mRNA was injected. Oocytes were incubated in Leibovitz’s L-15 medium (Life Technologies) with 3 mM L-glutamine, 2.5 mg/ml gentamycin, 15 mM HEPES (pH 7.4 with NaOH) until experiments.  

Electrophysiological Recordings and Data Analysis—One day after mRNA injection, oocytes were placed one at a time in a custom-built chamber (35), perfused with bath solution (in mM, 96 NaCl, 2 KCl, 1.8 CaCl2, and either 5 mM HEPES to pH 7.0, 7.4, 8.5, or 9.2 with NaOH/HCl or 5 mM MES to pH 5.8 with NaOH/HCl) for two-electrode voltage clamp experiments. L-glutamate (dissolved in bath solution) was applied for ~5 s with 1 min between subsequent applications using a ValveBank 8 perfusion system (AutoMate Scientific). Ivermectin was applied for longer until saturating current response was observed. Oocytes were clamped at −60 mV, and currents were recorded with microelectrodes filled with 3 M KCl, OC-725C amplifier (Warner Instruments), and Digidata 1550 digitizer (Molecular Devices) at 1 kHz with 200-Hz filtering. Peak current responses to L-glutamate were later analyzed in Clampfit 10 (Molecular Devices) with 10-Hz filtering for illustration. Peak current responses were plotted against glutamate concentration using the four-parameter Hill equation (tests described in Table 1) using Prism 6.

Amino Acid Sequence Alignment—Amino acid sequences were retrieved from UniProt. Brief names (as in Fig. 3A), full names, and UniProt sequence IDs (in parentheses) are as follows: GABAAR, human GABA_A receptor α1 subunit (P14867); RDL, Drosophila melanogaster RDL GABA receptor (P25123); GlyR, human glycine receptor α1 subunit (P23415); GluCl-A, Aplysia californica GluCl-2 (C7DLK0); GluCl-S, Chistothose mansoni GluCl-2.1 (T2CSA6); GluCl-D, D. melanogaster GluCl (Q94990); GLC-2, C. elegans GluCl β (Q17328); GLC-1, C. elegans GluCl α (G5EB3); and AVR14B, H. contortus AVR-14B GluCl (O46124). Sequences were aligned in MUSCLE (36) using default parameters in the European Bioinformatics Institute portal. For display in Fig. 3A, only three Loop G residues (AVR14B/O46124 residues 75–77) and five Loop D residues (AVR14B/O46124 residues 92–96) were shown.

Author Contributions—All authors conceptualized the design and developed the methodology. T. L. and V. V. K. performed investigations. T. L. wrote the original draft, and all authors reviewed and edited the manuscript; T. L. performed visualization. T. L. and S. A. P. were responsible for funding acquisition; S. A. P. supervised the study.

References


Arginine Side Chain in Ligand Recognition


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