Arg-274 and Leu-277 of the γ-Aminobutyric Acid Type A Receptor α2 Subunit Define Agonist Efficacy and Potency*

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Sean M. O'Shea§ and Neil L. Harrison‡§¶

From the ¶Departments of Neurobiology, Pharmacology, and Physiology and §Anesthesia and Critical Care, University of Chicago, Chicago, Illinois 60637 and ¶Department of Anesthesiology, Weill Medical College of Cornell University, New York, New York 10021

Alanine-scanning mutagenesis and the whole cell voltage-clamp technique were used to investigate the function of the extracellular loop between the second and third transmembrane domains (TM2–TM3) of the γ-aminobutyric acid type A receptor (GABA<sub>A</sub>-R). A conserved arginine residue in the TM2–TM3 loop of the GABA<sub>A</sub>-R α<sub>2</sub> subunit was mutated to alanine, and the mutant α<sub>2</sub>(R274A) was co-expressed with wild-type β<sub>1</sub> and γ<sub>2S</sub> subunits in human embryonic kidney (HEK) 293 cells. The GABA EC<sub>50</sub> was increased by about 27-fold in the mutant receptor relative to receptors containing a wild-type α<sub>2</sub> subunit. Similarly, the GABA EC<sub>50</sub> at α<sub>2</sub>(L277A)-β<sub>1</sub>γ<sub>2S</sub> and α<sub>2</sub>(R274A)β<sub>1</sub>γ<sub>2S</sub> GABA<sub>A</sub>-R combinations was increased by 51- and 4-fold, respectively. The α<sub>2</sub>(R274A) or α<sub>2</sub>(L277A) mutations also reduced the maximal response of piperidine-4-sulfonic acid relative to GABA by converting piperidine-4-sulfonic acid into a weak partial agonist at the GABA<sub>A</sub>-R. Based on these results, we propose that α<sub>2</sub>(Arg-274) and α<sub>2</sub>(Leu-277) are crucial to the efficient transduction of agonist binding into channel gating at the GABA<sub>A</sub>-R.

The GABA<sub>A</sub>-R receptor (GABA<sub>A</sub>-R) as well as the glycine (Gly-R) and nicotinic acetylcholine receptors belong to a homologous gene family of ligand-gated ion channels (1, 2). The GABA<sub>A</sub>-R is made up of five glycoprotein subunits that have been proposed to contain four transmembrane domains (TM1–TM4) with part of the TM2–TM3 loop of the GABA<sub>A</sub>-R α<sub>2</sub> subunit in the extracellular loop between the second and third transmembrane domains (9). The diagram displays the position of the α<sub>2</sub>(Arg-274) residue that is the primary focus of this study and is located within the predicted short extracellular loop between TM2 and TM3. The function of the TM2–TM3 loop in GABA<sub>A</sub>-R subunits is currently unknown, but the corresponding region of the Gly-R α subunit has been proposed to influence the efficiency of agonist-induced gating (10). A partial sequence alignment of Gly-R α1 compared with GABA<sub>A</sub>-R α<sub>2</sub>, β<sub>1</sub>, and γ<sub>2S</sub> subunits (Fig. 1B) illustrates the similarity between the amino acid sequences in the TM2–TM3 loop within the group of receptor subunits. We therefore predicted that mutations in this region of the GABA<sub>A</sub>-R will produce similar effects on receptor gating. GABA<sub>A</sub>-R amino acid residues α<sub>2</sub>(Arg-274), α<sub>2</sub>(Leu-277), and α<sub>2</sub>(Lys-279) are homologous to the residues in the Gly-R α1 subunit that alter the glycine EC<sub>50</sub> when mutated (Arg-271 (11), Leu-274 (12), and Lys-276 (13); Fig. 1B, boldface residues). R271Q, R271L, and K276E are naturally occurring point mutations that dramatically reduce glycine receptor sensitivity to agonist and are associated with the human neurological disorder hereditary hyperekplexia. These mutations increase the EC<sub>50</sub> and reduce the maximal response of the glycine receptor agonists taurine and β-alanine, although the mutations do not alter the binding of the competitive glycine receptor antagonist [3H]strychnine (12).

To explore whether these conserved arginine residues in the GABA<sub>A</sub>-R play a role in receptor function, we mutated each of the homologous arginines to alanine and determined the resultant GABA EC<sub>50</sub> (also known as potency or apparent affinity). In addition, we performed an alanine scan of the TM2–TM3 loop to identify other residues in the α subunit that may be important for GABA<sub>A</sub>-R function. Finally, we measured the effects of selected mutations on the maximal response (relative efficacy) of piperidine-4-sulfonic acid (P4S) relative to GABA.

EXPERIMENTAL PROCEDURES

Transient Receptor Expression—Wild-type or mutant receptor cDNA was expressed in human embryonic kidney (HEK) 293 cells via the pcIS2 vector (14). The GABA<sub>A</sub>-R α<sub>2</sub>(15), β<sub>1</sub>, γ<sub>2S</sub>, and γ<sub>2L</sub> subunits (17) cDNA that was used in this study was of human origin. The γ<sub>2S</sub> nomenclature refers to the shorter of two splice variants of the GABA<sub>A</sub>-R γ<sub>2L</sub> subunit; the longer variant is γ<sub>2L</sub>. The two splice variants differ only by the absence or presence of eight amino acid residues in the intracellular loop between the putative TM3 and TM4 transmembrane domains (17). Cells were transfected using the calcium phosphate precipitation technique (18) to achieve transient expression of human GABA<sub>A</sub>-R. Anesthesia and Critical Care, University of California, San Diego, CA. The abbreviations used are: GABA<sub>A</sub>-R, GABA<sub>A</sub>-R receptor; Gly-R, glycine receptor; P4S, piperidine-4-sulfonic acid; HEK, human embryonic kidney; TM, transmembrane domain.

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† To whom correspondence should be addressed: C. V. Starr Laboratory for Molecular Neuropharmacology, Dept. of Anesthesiology, Weill Medical College of Cornell University, 525 E. 68th St., New York, NY 10021. Tel.: 212-746-5325; Fax: 212-746-8479; E-mail: nelh2001@mail.med.cornell.edu.

§ The abbreviations used are: GABA<sub>A</sub>-R, GABA<sub>A</sub>-R receptor; Gly-R, glycine receptor; P4S, piperidine-4-sulfonic acid; HEK, human embryonic kidney; TM, transmembrane domain.
were confirmed by automated fluorescent DNA sequencing of the complete receptor subunit cDNA insert (University of Chicago DNA Sequencing Facility, Chicago, IL). For technical reasons, the GABA<sub>A</sub> γ<sub>2S</sub> subunit proved to be easier to mutate than the γ<sub>2</sub>L subunit, so a γ<sub>2S</sub>R296A mutation was used for this study.

**Tissue Culture—**HEK 293 cells (American Type Culture Collection, Manassas, VA) were cultured on poly-D-lysine-treated coverslips (Sigma) in Eagle's minimum essential medium (Sigma) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), L-glutamine (0.292 mg/ml, Life Technologies Inc.). HEK 293 cells were either dissolved directly into extracellular solution each day or were normalized to the maximal current that could be elicited by GABA-induced responses in each cell were normalized to the maximal current that could be elicited by GABA-induced responses in each cell.

**Data Analysis—**Concentration-effect data were fitted (Kaleidagraph, Reading, PA) with the following equation,

\[
\frac{[\text{drug}]}{[\text{agonist}]} = \frac{\text{IC}^{50}}{[\text{IC}^{50}]} + \frac{1}{[\text{IC}^{50}]} 
\]

(Eq. 1)

![Image](https://example.com/image.png)

**Fig. 1.** Putative GABA<sub>A</sub>-R α<sub>2</sub> subunit topology. Depicted in A are four transmembrane domains (TM1–TM4), one disulfide bond at the N terminus, two intracellular (IC) loops (TM1–TM2 and TM3–TM4), and one extracellular loop (TM2–TM3). GABA<sub>A</sub> α<sub>2</sub>(Arg-274) is shown in bold (TM2–TM3 loop, R274). Residues implicated in agonist binding are underlined. B, partial sequence alignment of GABA<sub>A</sub> and glycine receptor subunits with residues that alter agonist EC<sub>50</sub> shown in bold and labeled with asterisks.

**Fig. 2.** GABA EC<sub>50</sub> is higher at the mutant α<sub>2</sub>R274A/β<sub>2S</sub> than at wild-type α<sub>2</sub>β<sub>2</sub> GABA<sub>A</sub> receptors. GABA-induced responses from individual cells expressing either wild-type (a) or mutant (b) GABA<sub>A</sub> receptors are shown. Bars over current traces indicate the duration of GABA application with the concentration of applied agonist in µM. C, GABA concentration-effect curves for wild-type, α<sub>2S</sub>β<sub>1</sub>γ<sub>2S</sub>, α<sub>2</sub>R267A/β<sub>1</sub>γ<sub>2S</sub>, α<sub>2</sub>R274A/β<sub>1</sub>γ<sub>2S</sub>, and α<sub>2</sub>Sβ<sub>1</sub>γ<sub>2S</sub>(R296A) GABA<sub>A</sub> receptors. Data points shown are the means of multiple normalized experiments (see Table I). Bars indicate S.E.

where \(I_{\text{max,GABA}}\) is the fraction of the maximally obtainable GABA response, ic<sub>50</sub> is the concentration of agonist producing a half-maximal response, and \(n_{\text{h}}\) is the Hill coefficient. Agonist responses in each cell were normalized to the maximal current that could be elicited by GABA. Relative efficacy (e) was then determined by \(e = I_{\text{max,GABA}} / I_{\text{max,GABA}}\). Pooled data are presented as mean ± S.E. Statistical significance was determined at the p < 0.05 level by a one-way analysis of variance test with (Dunnnett’s post-hoc test except when the data did not fit a normal distribution. In those cases, a one-way analysis of variance test was performed on ranks with Dunnnett’s post-hoc test at the p < 0.05 level.

**Materials—**GABA and P4S (Research Biochemicals Inc., Natick, MA) were either dissolved directly into extracellular solution each day or stored overnight at 4 °C in sealed Nalgene<sup>®</sup> bottles. All other reagents were purchased from Sigma.

**RESULTS**

A comparison of the GABA responses of wild-type α<sub>2</sub>β<sub>1</sub>γ<sub>2S</sub> GABA<sub>A</sub> receptors (Fig. 2A) and mutant α<sub>2</sub>S(R274A)β<sub>1</sub>γ<sub>2S</sub> receptors (Fig. 2B) in HEK 293 cells shows that brief applications of agonist to either receptor combination produced saturable concentration-dependent inward currents. The amplitudes of the maximal GABA response were similar in wild-type and mutant receptors (data not shown). However, inspection of the pooled data (Fig. 2C and Table I) revealed that α<sub>2</sub>S(R274A)β<sub>1</sub>γ<sub>2S</sub> receptors gave a 27-fold increase in the GABA EC<sub>50</sub> indicating that the mutant receptors were significantly less sensitive to GABA than the wild-type receptors.
We then characterized the GABA responses of receptors containing β1(R267A) or γ2L(R296A) subunits that were again co-expressed with the appropriate wild-type subunits. Both mutant subunits (similar to the α2(R274A) subunit) were incorporated into functional GABA_α receptors, but neither mutation produced significant shifts in the GABA EC50 (Fig. 2C). The long splice variant of the γ2 subunit was used as a background to make the mutant γ2L(R296A) subunit. Therefore, the GABA EC50 for wild-type α2β1γ2L receptors is included in Table I for comparison. There was no statistical difference between the GABA EC50 values for receptors containing either the γ2s or γ2l splice variant, and the α2β1γ2s(R296A) GABA EC50 was not significantly different from wild-type α2β1γ2L receptors.

We next examined the α2(Arg-274) residue in greater detail to determine the effects of different amino acid substitutions on the GABA EC50. We chose mutations at this position that would neutralize a positive charge (R274Q), replace a positive R274D, or make a conservative replacement (R274K). GABA_α receptors containing α2(R274Q) or α2(R274D) exhibited smaller (7-fold) changes in GABA EC50 than receptors containing α2(R274A) (Fig. 3). In contrast, the GABA EC50 was not significantly altered at α2(R274S)γ2s or α2(R274K)γ2s receptors.

After focusing on the α2(Arg-274) position, we investigated whether the mutation of neighboring residues within the TM2–TM3 extracellular loop would produce similar alterations in GABA EC50. Using alanine-scanning mutagenesis, we discovered two more substitutions that decreased GABA sensitivity. GABA α2(L277A)β1γ2s receptors showed a 51-fold increase in GABA EC50 when compared with wild-type receptors, and α2(R279A) showed a 4-fold increase whereas α2(P278A) did not alter the GABA EC50 (Fig. 4). Other mutations produced no changes in GABA EC50 (Table I).

In the simplest model of receptor occupancy and gating (21) at least two sequential events must occur prior to receptor activation, each one determined by the ratio of individual microscopic rate constants as shown in Scheme 1. First, the agonist (A) must bind to the receptor (R), and second, the agonist-receptor complex (AR) must isomerize into the open state (AR⁎). Therefore, GABA EC50 may be influenced at either step in the receptor activation pathway. As a result, steady-state measurements of GABA EC50 or [3H]GABA binding alone will not determine whether a mutation in the receptor affects the affinity of agonist binding per se or alters receptor gating efficiency from an allostERIC site (22).

To address this issue, we used a traditional partial agonist approach (23–26) to determine whether gating of the GABA_α-R is impaired in α2(R274A)γ2s receptors. Rather than individually determining each of the rate constants shown in Scheme 1, we compared the e of the fractional maximal response versus GABA of P4S (27, 28) at wild-type and mutant receptors. Because the agonist relative efficacy is directly dependent on the isomerization process (β/α, Ref. 22), we predict that a change in gating should be accompanied by an alteration in the relative efficacy of a partial agonist.

P4S produced responses in wild-type α2β1γ2 L receptors (Fig. 5A) (Table II) that were similar in maximal amplitude to GABA responses (Fig. 5A, last trace). In contrast, α2(R274A)β1γ2s receptors exhibited maximal P4S responses that were on average about half of the amplitude of the maximal current
TM2–TM3 Loop of the GABAA-R α2 Subunit

FIG. 5. P4S has a lower relative efficacy and higher EC50 at α2(R274A)β1γ2S than wild-type α2β1γ2S receptors. P4S-induced responses from individual cells expressing either wild-type (A) or α2(R274A)β1γ2S (B) GABAA receptors are shown. Bars indicate S.E. C, P4S concentration–effect curves for both wild-type and α2(R274A)β1γ2S receptors. Bars indicate S.E.

DISCUSSION

Using alanine-scanning mutagenesis, we have discovered three amino acid residues in the TM2–TM3 loop of the GABAA-R α2 subunit (Arg-274, Leu-277, and Lys-279) that are involved in determining the EC50 of GABA. Our results imply specific structural and functional roles for these residues because alanine replacement of neighboring residues in the TM2–TM3 loop of the GABAA α2 subunit (N275A, S276A, P278A, V280A, and Y282A) produced no change in the GABA EC50.

P4S is a partial agonist at α1-containing GABAA receptors (26, 29) but showed a high relative efficacy at receptors containing either a wild-type α2 subunit or α2(P278A), which is a mutant subunit that produced no effect on GABA EC50. In contrast, P4S showed a marked reduction in relative efficacy at receptors containing α2(R274A) or α2(L277A). We have therefore demonstrated a clear association between an increase in GABA EC50 and a reduction in P4S relative efficacy. Mutations that alter agonist binding alone produce lateral shifts in the concentration–effect curve with no change in relative efficacy (22, 30). A mechanism in which the α2(R274A) and α2(L277A) mutations impair receptor isomerization is more consistent with a simultaneous increase in GABA EC50 and P4S EC50 and a decrease in P4S relative efficacy. However, we cannot rule out a simultaneous decrease in both binding affinity and gating efficiency.

The contribution of the α subunit to the GABA binding site has been incompletely mapped. To date, α1(Phe-64) is the only residue in the GABAA-R that has been proven to interact directly with the agonist muscimol (using photoaffinity labeling, see Ref. 31). However, receptors containing mutant α1(F64C), α1(R66C), or α1(S68C) are protected from modification by sulfhydryl reagents in the presence of GABA making Arg-66 and Ser-68 good candidates as additional GABA contact points (33). Other N-terminal residues of the α1 subunit, such as Arg-120 (33) and Ile-121 (34), have also been proposed to form part of the agonist binding site, but the evidence has been less direct. In those studies, α1(R120K) or α1(I121V) mutations produced dramatic increases in the EC50 of GABA and decreases in the binding of the competitive antagonist [3H]SR 95531.

In contrast, a mutation that alters gating rather than binding should produce no change in antagonist affinity because it does not induce a conformational shift to the open state. Our results with α2(Lys-279) are complemented by those of a recent study (35) that reports that oocytes expressing rat α2(K278A), β2, and γ2S GABAA-R subunits show a 6-fold increase in GABA
EC\textsubscript{50}. However, a Schild analysis shows no change in bicuculline affinity. Because GABA, P4S, and bicuculline all presumably compete at the GABA binding site, it seems unlikely that α\textsubscript{2}(Arg-274), α\textsubscript{2}(Leu-277), and α\textsubscript{2}(Lys-279) actually function as agonist contact points on the GABA\textsubscript{A}-R α subunit. We propose instead that the TM2–TM3 loop region of the GABA\textsubscript{A}-R α subunit plays a critical role in receptor gating.

What physical characteristics of the amino acid side chain at position α\textsubscript{2}(Arg-274) are associated with efficient GABA\textsubscript{A}-R gating? The results of this study suggest that no simple correlation exists between the GABA EC\textsubscript{50} and either the volume, polarity, or hydrophobicity of the residue at this position.

Do these TM2–TM3 loops act as “hinges” (12) that limit the mobility of the TM2 transmembrane region in the presence of agonist? The TM2 domain of the GABA\textsubscript{A}-R, by analogy with the M2 domain of other members of the ligand-gated ion channel superfamily (36–38), is likely to be an α helix. In support of this prediction, the substituted cysteine accessibility method reveals that every third or fourth amino acid residue of the GABA\textsubscript{A}-R α subunit is accessible to a water-soluble alkylation reagent (4). Conversely, within residues 59–68 of the GABA\textsubscript{A}-R α subunit, every second residue is accessible, thus providing evidence that this region is a β-strand (32). Unfortunately, no such structural information is yet available with the TM2–TM3 loop.

Based on the similarity between the amino acid sequences of the GABA\textsubscript{A} and glycine receptor subunits, it is possible that the TM2–TM3 extracellular loop plays a similar role in GABA\textsubscript{A} and glycine receptor function (12, 39). One laboratory (13) has used single channel analysis of α\textsubscript{1}(K273E)β glycine receptors to measure an increase in the closing rate constant (Scheme 1, α), whereas other researchers have shown that α\textsubscript{1}(R271Q) in the Gly-R reduces the single-channel conductance (25, 40). Naturally occurring mutations of the TM2–TM3 loop in muscle nicotinic receptor subunits, which produce the slow-channel form of myasthenia gravis (41), alter the efficacy of the partial agonist choline by increasing the opening rate of the receptor (Scheme 1, β). The TM2–TM3 loop may therefore also play a role in the gating of other ligand-gated ion channels within this gene family.

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