Locating the Carboxylate Group of GABA in the Homomeric rho GABA\(_A\) Receptor Ligand-binding Pocket*

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\(\gamma\)-Aminobutyric acid, type A (GABA\(_A\)) receptors, of which the GABA\(_C\) receptor family is a subgroup, are members of the Cys loop family of neurotransmitter receptors. Homology modeling of the extracellular domain of these proteins has revealed many molecular details, but it is not yet clear how GABA is orientated in the binding pocket. Here we have examined the role of arginine residues that the homology model locates in or close to the binding site of the GABA\(_C\) receptor (Arg-104, Arg-170, Arg-158, and Arg-249) using mutagenesis and functional studies. The data suggest that Arg-158 is critical for GABA binding and/or function; substitution with Lys, Ala, or Glu resulted in a nonfunctional receptors, and modeling placed the carboxylate of GABA within 3 Å of this residue. Substitution of Arg-104 with Ala or Glu resulted in >10,000-fold increases in EC\(_{50}\) values compared with wild type receptors, and modeling indicated a role of this residue both in binding GABA and in the structure of the binding pocket. Substitution of Arg-170 with Asp or Ala yielded nonfunctional receptors, whereas Lys caused an ∼10-fold increase in EC\(_{50}\). Arg-249 was substituted with Ala, Glu, or Asp with relatively small (∼4–30-fold) changes in EC\(_{50}\). These and data from other residues that the model suggested could interact with GABA (His-105, Ser-168, and Ser-243) support a location for GABA in the binding site with its carboxylate pinched between Arg-158 and Arg-104, with Arg-104, Arg-170, and Arg-249 contributing to the structure of the binding pocket through salt bridges and/or hydrogen bonds.

GABA\(_C\), the major inhibitory neurotransmitter in the mammalian central nervous system (1). It mediates its effects via both ionotropic (GABA\(_A\)) and metabotropic (GABA\(_B\)) receptors. GABA\(_C\) receptors are a subfamily of GABA\(_A\) receptors and are sometimes referred to as GABA\(_A\) receptors, because the first subunit identified from this class of receptor was called p1 (2). These receptors have been separated from “classic” GABA\(_A\) receptors because of their distinct pharmacological properties; GABA\(_C\) receptor-mediated responses are not inhibited by bicuculline (the classic competitive GABA\(_A\) receptor antagonist) nor induced by baclofen (the classic GABA\(_B\) receptor agonist). In addition, 3-aminopropylphosphinic acid, a potent competitive GABA\(_C\) receptor antagonist, acts as an agonist at the GABA\(_B\) receptor and is inactive at the GABA\(_A\) receptor, and GABA and trans-4-aminocrotonic acid are more potent agonists at GABA\(_C\) receptors than GABA\(_A\) or GABA\(_B\) receptors (3). Thus there is clearly some variation in the structures of the different GABA-binding sites contained in these receptors, although the sequence homology between GABA\(_A\) and GABA\(_C\) receptor subunit sequences (>25%) indicates that their basic structures will be similar (structural homology is estimated at close to 80%) and will be similar to other members of the Cys loop family (4, 5).

The lack of detailed structural information available for Cys loop receptors means that homology modeling is currently one of the best approaches to investigate possible molecular interactions between binding site residues and ligands. This approach has been made possible by the availability of the high resolution structure of the acetylcholine binding protein (AChBP), which is homologous to the extracellular domain of the nicotinic ACh receptor (6). Using this as a template, computer-generated models of ligand-binding pockets of Cys loop receptors, combined with previous data from structure-activity studies, have identified important features of these pockets and on the orientation of agonists and antagonists when located in their binding sites (7–13). Generating an accurate representation of the orientation of GABA in the GABA\(_C\) receptor-binding site could identify the processes involved in ligand recognition at this receptor and would also assist in drug design; current data indicate that drugs acting on GABA\(_C\) receptors could possibly be used to treat visual, sleep, and cognitive disorders (4, 14).

It has recently been shown that Tyr-198 forms a cation-π interaction with the positive amine of GABA (15), and thus we can confidently locate this part of the GABA molecule close to this residue. The aim of this study was to locate the other end of GABA, the carboxylate group, in the binding site. This negatively charged end of GABA has been shown to interact with one or more positively charged residues in the GABA\(_A\) receptor-binding pocket (16), and here we examine the role of positively charged and polar residues that are in or close to GABA in the GABA\(_C\) receptor-binding site (Fig. 1). The data have allowed us to define the probable orientation of GABA.

**EXPERIMENTAL PROCEDURES**

**Materials**—The human GABA\(_{p1}\) subunit was kindly gifted by D. S. Weiss. All of the cell culture reagents were obtained from
invitrogen (paisley, UK), except fetal calf serum, which was from labtech international (Ringmer, UK). GABAC receptor antiserum was from Santa cruz Biotechnology, Inc. All of the other reagents were of the highest obtainable grade.

**Oocyte Preparation**—This was as described previously (17). Harvested stage V-VI XENOPUS oocytes were washed in four changes of OR2 (82.5 mM NaCl, 2 mM KCl, 1 mM MgCl2, 5 mM HEPES, pH 7.5), defolliculated in 1 mg/ml collagenase for ~1 h, washed again in four changes of OR2, and transferred to 70% LEIBOVITZ media (invitrogen) buffered with 10 mM HEPES, pH 7.5. The following day they were injected with 5 ng of mRNA produced by in vitro transcription using the mMESSAGE mMACHINE kit (Ambion) from wild type or mutant DNA (the latter generated using the KUNKEL method (18)). Electrophysiological measurements were performed 24–72 h post-injection.

**Electrophysiological Recordings**—Two-electrode voltage clamp of XENOPUS oocytes was performed using standard electrophysiological procedures as previously described (17). Briefly, a GeneClamp 500B amplifier was connected to a PC running CLAMPEx v6.0.3 software via a DigiData1200 Series Interface (all from Axon Instruments, Inc.). Glass microelectrodes were pulled from GC150TF-10 glass capillaries (Harvard Apparatus) using a P-87 micropipette puller (Sutter) to a resistance of 0.5 MΩ and back-filled with 3 M KCl. The oocytes were maintained at a holding potential of −50 mV unless stated otherwise and perfused continuously with CFFR (calcium-free frog ringer; 115 mM NaCl, 2.5 mM KCl, 1.8 mM MgCl2, 10 mM HEPES, pH 7.5) at a rate of 3–4 ml/min. GABA was diluted in CFFR and applied to the bath using a Valve Bank 8 II system (Automate Scientific, Inc.). Concentration-response curves and parameters were obtained using Prism software (GraphPad, San Diego, CA).

**Cell Culture**—Human embryonic kidney (HEK) 293 cells were maintained on 90 mm tissue culture plates at 37 °C and 7% CO2 in a humidified atmosphere. They were cultured in dulbecco’s modified eagle’s medium/nutrient mix F-12 (1:1) with GLUTAMAX I™ containing 10% fetal calf serum and passaged when confluent. The cells grown on 22-mm coverslips were

![FIGURE 1. A, adjacent subunits (principal and complementary) showing the positions of the Arg residues and Tyr-198 in a homology model of the GABAC receptor extracellular domain. Only two of the five subunits have been shown for ease of viewing, B, alignment of representative GABAC, 5-HT3A, AChBP, GABAA, and glycine receptor subunit sequences. The binding loops of the receptors are indicated by black lines above the alignment. The Arg residues in the binding pocket are in a black box.](image-url)
transfected using calcium phosphate precipitation (19) at 80–90% confluency and incubated for 3–4 days before assay.

**Immunofluorescent Localization**—The methodology was similar to that described for localization of the 5-HT3 receptor, as described previously (17). Briefly, transfected cells were fixed in ice-cold 4% paraformaldehyde and then incubated with the GABA<sub>C</sub>/H<sub>9267</sub> antisera (which recognizes the extracellular domain) at 1:100 dilution. Biotinylated anti-rabbit IgG (Vector) and fluorescein isothiocyanate avidin D (Vector) were used to detect bound antibody as per the manufacturer's instructions. The coverslips were mounted in Vectashield mounting medium (Vector), and immunofluorescence was observed using a Nikon optiphot or confocal microscope.

**Modeling and Ligand Docking**—The protein sequence of the extracellular domain of the human GABA<sub>C</sub>/H<sub>9267</sub> receptor was aligned with the sequence of AChBP from <i>Lymnaea stagnalis</i> using FUGUE, and a three-dimensional homology model was generated using MODELLER 6v2 based on the crystal structure of AChBP at 2.7 Å resolution (13). The pentamer was generated by superimposing the model onto each protomer of AchBP, and the generated pentamer model was then energy-minimized using the force field implemented in MODELLER 6v2. The best model was selected after Ramachandran plot analysis of all of the generated models. Docking was carried out using GOLD 3.0 (The Cambridge Crystallographic Data Centre, Cambridge, UK).

**RESULTS**

**Functional Studies of Wild Type and Mutant GABA<sub>C</sub> Receptors**—<i>Xenopus</i> oocytes injected with wild type GABA<sub>C</sub>/H<sub>9267</sub> receptor subunit mRNA showed robust responses to GABA application 2–4 days after injection (Fig. 2). Dose-response curves revealed an EC<sub>50</sub> of 1.37 ± 0.18 μM and a Hill coefficient of 1.6 ± 0.25 (Table 1). These data are in good agreement with the values previously published (20, 21).

**Arginine Residues in the Binding Site**—R104A and R104E mutant receptors were functional, with high EC<sub>50</sub> values: 15,000 and 40,000 μM, respectively (Table 1). No responses were obtained from oocytes injected with mRNA for R104K at GABA concentrations up to 30,000 μM. Similarly, oocytes

| Receptor    | EC<sub>50</sub> (GABA) | Hill coefficient |
|-------------|----------------|-----------------|
| Wild type   | 1.37 ± 0.18       | 1.60 ± 0.25     |
| Arginine mutants |
| R104A       | 14430 ± 2939<sup>a</sup> | 0.95 ± 0.13     |
| R104E       | 44240 ± 11660<sup>a</sup> | 1.31 ± 0.31     |
| R104K       | NR<sup>a</sup>     |                 |
| R158A       | NR                |                 |
| R158E       | NR                |                 |
| R158K       | NR                |                 |
| R170A       | NR                |                 |
| R170D       | NR                |                 |
| R170K       | 9.48 ± 2.73<sup>a</sup> | 0.89 ± 0.18     |
| R249A       | 21.0 ± 1.30<sup>a</sup> | 1.18 ± 0.17     |
| R249K       | 37.98 ± 7.23<sup>a</sup> | 1.24 ± 0.23     |
| R249D       | 5.55 ± 0.97<sup>a</sup> | 1.37 ± 0.25     |
| Other mutants |
| H105A       | 3.63 ± 0.23<sup>a</sup> | 1.43 ± 0.11     |
| H105D       | 2.93 ± 0.55<sup>a</sup> | 0.99 ± 0.16     |
| H105R       | 69.39 ± 8.94<sup>a</sup> | 1.06 ± 0.12     |
| S168A       | 49.91 ± 1.63<sup>a</sup> | 1.37 ± 0.17     |
| S168T       | 13.13 ± 1.63<sup>a</sup> | 1.65 ± 0.27     |
| S168V       | NR                |                 |
| S243A       | 3.34 ± 0.34<sup>a</sup> | 1.35 ± 0.16     |
| S243T       | 34.14 ± 5.48<sup>a</sup> | 1.18 ± 0.18     |

<sup>a</sup> Significantly different from wild type (p < 0.05).
<sup>b</sup> NR, no response detected (GABA applied up to 30,000 μM; n = 4–8).
injected with mRNA from all of the receptors mutated at Arg-158 showed no responses to GABA at concentrations up to 30,000 μM. Oocytes from the same batches injected with wild type GABA mRNA showed expected responses, indicating that receptor expression in these oocytes was unaffected. R170A or R170D mutant receptors were also insensitive to GABA, but R170K mutant receptors gave rise to responses with an EC50 less than 10-fold different from wild type receptors. Arg-249 mutant receptors had relatively small increases in EC50 values (4–30-fold). Hill coefficients were not significantly changed for any of these receptors when compared with wild type receptors. Representative traces are shown in Fig. 2.

Other Polar Residues—His-105 is another potentially positively charged residue that might interact with GABA and is located in the binding pocket. However, our data showed that an interaction with this residue was unlikely, because all His-105 mutants tested were functional. H105A and H105D mutants had small increases in EC50 compared with wild type receptors (3–4-fold), although there was a larger increase (≈60-fold) in EC50 for H105R mutants. Thus size appears more critical than charge here. Hill coefficients were not significantly changed.

Two serine residues (Ser-168 and Ser-243) are located in the binding site and therefore may contribute to GABA stabilization via hydrogen bonds. Application of GABA to oocytes injected with mRNA for S168T and S168A mutant receptors revealed increases in EC50 of ≈10- and 50-fold, respectively, compared with wild type, whereas oocytes injected with mRNA for the S168V receptor showed no responses to GABA up to 30,000 μM. In the study of Ser-243 mutant receptors, increases in EC50 of ≈30- and ≈3-fold were observed for Thr and Ala mutants, respectively, compared with wild type. Hill coefficients were not significantly changed for any of the functional serine mutants.

Immunofluorescent Localization of Wild Type and Mutant Receptors—No function was observed for R104K, R170A, R170D, R158A, R158E, R158K, and S168V mutant receptors. This indicated that these changes either affected receptor binding, gating, and/or expression. All of the nonfunctional mutants localized to the surface of HEK293 cells following transient transfection (examples are shown in Fig. 3), suggesting that global receptor folding and assembly are not significantly affected. Those receptors that reach the cell surface are presumed to be pentamers, because single Cys loop receptors subunits that do not assemble in the ER are normally degraded (22). This assay was performed in mammalian cells, because there are problems from endogenous oocyte fluorescence. However, because mammalian cells are generally less tolerant of expression of ion channel proteins than oocytes, it is reasonable to assume that any receptors localized to the cell surface in HEK293 cells will also reach the surface in oocytes (17).

Modeling—The model revealed a GABA-binding pocket rich in aromatic residues, and in particular there was an aromatic box, consisting of Tyr-102, Tyr-198, Tyr-241, and Tyr-247, at one end of the binding site. Docking, combined with previous functional studies (15), suggest that the amine of GABA is located here. There are also a number of charged and hydrophilic residues in the binding pocket that have the potential to interact with GABA: Arg-104, His-105, Met-156, Arg-158, Ser-168, Ser-197, Ser-243, and Arg-249. Docking indicated that the carboxylate end of GABA was located close to Arg-104 and Arg-158 (Fig. 4).
In this study we have examined the role of the positively charged arginine residues located in the binding pocket of the GABA<sub>C</sub> receptor to define the orientation of GABA in the binding pocket. The most sensitive of these residues was Arg-158, because none of the amino acids that replaced it resulted in functional receptors. An Arg at position 104 is also important, because receptors mutated at this location were either insensitive to GABA, or their EC<sub>50</sub> values were increased more than 10,000-fold compared with wild type. At position 170 a positively charged residue appeared required for function, whereas a variety of residues were reasonably well tolerated at position 249. If we assume that the positively charged amine of GABA is close to Tyr-198 as previously proposed (14), these new data are consistent with the negatively charged carboxylate being located between Arg-104 and Arg-158, although closer to the latter (Fig. 4). Examination of the model suggests that Arg-104, Arg-170, and Arg-249 may also contribute to the stability of the binding site via hydrogen bonds and/or salt bridges. Specific interactions are described in more detail below.

Arg-104—The large changes in EC<sub>50</sub> following mutation at position 104 indicates that this Arg plays an important role in the function of the receptor. Because an EC<sub>50</sub> combines binding affinity and functional efficacy, it is not possible to tell from these data which is being affected. However, there is good evidence from GABA<sub>A</sub> receptor studies that this residue is involved specifically in binding. In the GABA<sub>A</sub> receptor α5 subunit (23), mutation to Lys of the equivalent arginine (Arg-70) caused an ~700-fold increase in EC<sub>50</sub>, but there was no change in diazepam and allopregnanolone modulation, and pentobarbital stimulation properties were unaffected, suggesting a specific alteration to GABA binding. Also, substituted cysteine accessibility method studies in the GABA<sub>A</sub> receptor α1 subunit have shown that methylthiosulphonate modification of the Cys derivative of Arg-66 (equivalent to Arg-104) is slowed by the presence of GABA or the competitive antagonist SR-95531, indicating a specific interaction of this residue with ligand (24, 25). Examination of the GABA<sub>C</sub>-binding site model suggests that Arg-104 is well placed to play a similar role here, because it could help to stabilize the positive charge of GABA. In addition the model suggests that it may have a role in contributing to a network of hydrogen bonds with the stretch of amino acids located immediately behind the binding pocket (Fig. 5A). This might explain the loss of function when Arg was replaced by Lys but not by Glu; manipulation of the model at this position using Swisspdb-viewer indicates that Lys would form fewer hydrogen bonds than either Arg and Glu and thus may subtly disrupt the binding pocket and/or modify the conformational change subsequent to binding. Thus we propose that this residue is critical for the formation and/or stability of the binding site and also contributes directly to GABA binding.

Arg-158—The lack of function when Arg-158 was mutated indicates that this residue is essential for receptor structure and/or function. However, because the Arg-158 mutants reached the plasma membrane in HEK293 cells, it suggests the global structure of the receptor is not changed and that this residue has a specific role in the microstructure of the binding...
The GABA<sub>C</sub> Receptor-binding Site

site or in receptor binding or function. An Arg is conserved here in all GABA<sub>A</sub>, GABA<sub>C</sub>, and glycine receptor subunits, suggesting an important role in all anion selective Cys loop receptors, and there is evidence that this role, at least for GABA<sub>A</sub> receptors, is in ligand binding. Mutation of this residue in the α5 subunit resulted in an ~300-fold increase in EC<sub>50</sub> but there was no change in diazepam and allopregnanolone modulation, and pentobarbital stimulation properties were unaffected, suggesting a specific alteration of GABA binding (22), although cysteine substitution at the equivalent Arg in the α1 subunit yielded receptors with expression that was too low to obtain data (5). The GABA<sub>C</sub> receptor model shows that this residue is 2.5 Å from the carboxylate of GABA and could stabilize GABA by both charge and hydrogen bond interactions. The model shows no specific interactions with any residue in the binding site (Fig. 5B), which would be energetically unfavorable; thus it is likely that this Arg interacts with water and/or with ligand. Because there is insufficient space in our model for water here when GABA is in the binding pocket, we propose that this residue is specifically involved in GABA binding.

Arg-170—The model suggests that Arg-170 could participate in both hydrogen bonds and salt bridges with two other regions of the receptor subunit (Fig. 5C). This hypothesis is not inconsistent with the data, because we observed functional receptors with Lys, which could compensate for both of these bonding requirements, but not Ala or Asp. These data might also suggest that a positive charge is necessary for receptor function at position 170, but such a hypothesis is not supported by the results of Sedelnikova et al. (5), in which a cysteine substitution yielded a functional receptor. The EC<sub>50</sub> for the R170C mutant was increased ~10-fold compared with wild type, a shift similar to that we observed for the R170K receptor. Because Cys can form hydrogen bonds, it could compensate for some extent for the loss of Arg. It is not possible to tell from our data whether binding affinity or functional efficacy is being affected, but the study of Sedelnikova et al. (5) indicated that this is a ligand-binding residue, because the Cys mutant at this position was protected from modification by both GABA and a competitive GABA<sub>C</sub> receptor antagonist. Further evidence comes from GABA<sub>A</sub> receptor work, where the equivalent residue (Arg-131) has been proposed to form part of a crown of arginines (in addition to Arg-207 and Arg-66) that stabilize the GABA carboxylate (16). Interestingly our model of the GABA<sub>C</sub> receptor-binding site suggests that Arg-170 may be too far from GABA to interact with it, although it is possible that this region of loop E may be closer to GABA than predicted. Interestingly, the recent cryo-electron microscope images of the nicotinic ACh receptor (26) show that structural variations exist in the β5-β5’ stretch (located in loop E) between the ligand-bound AChBP and its unbound state, suggesting that movement does occur in this region on agonist binding. More structural data are needed to prove this hypothesis, but the current model and data both indicate an important role of Arg-170 either with adjacent residues that could be important to maintain the structure of the binding region and/or as a residue directly involved in GABA binding.

Arg-249—The final arginine investigated was Arg-249. A charged residue is conserved at this position in many members of the Cys loop family and has been shown to be important in 5-HT<sub>3</sub> and GABA<sub>A</sub> receptors (16, 27). Indeed, Wagner et al. (16) proposed that the equivalent Arg-207 in the GABA<sub>A</sub> receptor makes direct contact with the ligand and is partially responsible for satisfying the negative charge of the carboxylate of GABA. If this was also true for the GABA<sub>C</sub> receptors, then large increases in EC<sub>50</sub> values would be expected upon Arg-249 mutation. Significant differences in EC<sub>50</sub> value compared with wild type were recorded for the R249A and R249K mutants, yet these changes were smaller (~15- and 25-fold increases, respectively) than those observed for the GABA<sub>A</sub> R207C mutation (~70-fold increase for R207C (28)) and much smaller for those observed for Arg-158 and Arg-104 mutants, suggesting that this hypothesis is not correct. Without better structural data and/or an understanding of the conformational changes that result in channel opening, it is difficult to explain these apparent differences in relative EC<sub>50</sub> values between putative ligand-binding residues, but we might speculate that the very large changes observed when GABA<sub>C</sub> receptor residues are mutated might be due to it having fewer residues that participate in the binding interaction, and particularly the interaction that stabilizes the positive charge on the GABA molecule. Certainly our data indicate that the GABA<sub>C</sub> receptor has no preference for a charged residue at position 249, and thus it is clearly not required in the GABA<sub>C</sub> receptor to satisfy the negative carboxylate of GABA. The model suggests that the primary role of this residue maybe to assist the formation of the correct structure of the binding pocket by hydrogen bonding with Tyr-241 (Fig. 5D), and we currently favor this proposal.

Other Residues—Two other Arg residues are within 10 Å of the binding pocket (Arg-144 and Arg-221). Previous studies have shown that mutation of either of these amino acids to cysteine does not change the EC<sub>50</sub> of the receptor and that such mutants are not accessible to sulphydryl-specific reagents (5). Thus it seems reasonable to assume that these arginines do not interact with ligand.

It is also possible that other positively charged residues may contribute to ligand binding. His-141, Lys-143, and Lys-217 have been investigated using the substituted cysteine accessibility method (5), and these data suggested that they were not protected from modification by GABA and thus were not present in the binding site. Another possibility is His-105, but we observed only small increases in EC<sub>50</sub> when this was mutated to Ala or Asp, although the larger Arg did increase EC<sub>50</sub> by ~60-fold, suggesting that size and not charge is the critical factor at this location. This supports the model, and previous studies in GABA<sub>A</sub> and 5-HT<sub>3</sub> receptors, which all predict this region to be β-sheet, with His-105 or its equivalent facing away from the pocket (24).

In addition to interaction with positive residues, the carboxylate of GABA may also be stabilized through hydrogen bonds to other polar residues in the binding pocket. If the docking pose showing the carboxylate near to Arg-158 and Arg-104 is accurate, then Ser-168 and Ser-243 are in sufficiently close proximity to interact with the ligand. To investigate this, each of these residues was mutated such that the hydrogen-bonding
moiety was either conserved or removed. These data revealed that Ser-243 does not form such a bond through its side chain (replacement with Thr was much less favorable than with Ala), but a hydroxyl group was favored at position 168. Thus a hydrogen bond here may help to stabilize GABA or may contribute to maintaining the structural integrity of the binding pocket.

Conclusions—Our data suggest that Arg-158 and Arg-104 are most closely associated with the ligand in the GABAC receptor, with Arg-158 having the most important role. These two positive amino acids could “pincer” the negative carboxylate, satiating its charge. Such an orientation is predicted by studies docking GABA into a model of the ρ1 extracellular domain. In this orientation the cation–π interaction with Tyr-198 is also satisfied, offering further support for this docking pose (Fig. 4). Thus our study has provided experimental evidence for a location of GABA in which potential partners for both the positive and negative groups of GABA are provided and also suggests that binding of GABA to the GABAC receptor may differ subtly from that in the GABAA receptor.

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