The glycine receptor is a member of the ligand-gated ion channel receptor superfamily that mediates fast synaptic transmission in the brainstem and spinal cord. Following ligand binding, the receptor undergoes a conformational change that is conveyed to the transmembrane regions of the receptor resulting in the opening of the channel pore. Using the acetylcholine-binding protein structure as a template, we modeled the extracellular domain of the glycine receptor α1-subunit and identified the location of charged residues within loops 2 and 7 (the conserved Cys-loop). These loops have been postulated to interact with the M2–M3 linker region between the transmembrane domains 2 and 3 as part of the receptor activation mechanism. Charged residues were substituted with cysteine, resulting in a shift in the concentration-response curves to the right in each case. Covalent modification with 2-(trimethylammonium)ethyl methanethiosulfonate was demonstrated only for K143C, which was more accessible in the open state than the closed state, and resulted in a shift in the EC_{50} toward wild-type values. Charge reversal mutations (E53K, D57K, and D148K) also impaired channel activation, as inferred from increases in EC_{50} values and the conversion of taurine from an agonist to an antagonist in E53K and D57K. Thus, each of the residues Glu-53, Asp-57, Lys-143, and Asp-148 are implicated in channel gating. However, the double reverse charge mutations E53K:K276E, D57K:K276E, and D148K:K276E did not restore glyicine receptor function. These results indicate that loops 2 and 7 in the extracellular domain play an important role in the mechanism of activation of the glycine receptor although not by a direct electrostatic mechanism.

Fast synaptic transmission in the central nervous system is mediated by members of the ligand-gated ion channel (LGIC) receptor superfamily. The glycine receptor (GlyR) is a member of the nicotinic-like LGIC superfamily that includes the nicotinic acetylcholine (nAChR), serotonin type 3 (5-HT_{3R}), and γ-aminobutyric acid (GABA_{A}R) receptors (1, 2). Each of these receptors is pentameric complexes arranged around a central ion conducting pore. Individual subunits share a similar membrane topology, with hydrophobic complexes arranged around a central transmembrane domain at the N terminus and four putative transmembrane domains (M1–M4) (3). A key characteristic of these receptors is the integral ion channel that is opened following ligand binding. The extracellular domain contains the ligand binding site, which is spatially separate from the M2 domain that lines the ion channel pore of these receptors (3, 4). While there is an accumulated body of data on the structures of these receptors, relatively little is known about the structures that may link these two spatially separate domains to effect receptor activation.

Inherited mutations located in the regions flanking the M2 domain of the GlyR α1-subunit are associated with human startle disease (hyperekplexia) (5) and startle syndromes in other species (6). In humans, the startle mutations R271L/Q and K276E map to the M2–M3 linker domain (5, 7). At both locations, the mutations result in the substitution of a positive charged residue. The effect of these mutations is to disrupt signal transduction, as inferred from the increases in EC_{50} for glycine and the conversion of taurine from an agonist to an antagonist (8). This was also demonstrated at the single channel level for the K276E mutation (9). An inherited mutation in the M2–M3 region of the nAChR is associated with a form of congenital myasthenic syndrome (10) and a mutation in the GABA_{A}R is associated with a rare form of epilepsy (11), both of which disrupt channel gating. In addition, site-directed mutagenesis and covalent modification studies have demonstrated that the M2–M3 region undergoes a conformational change that is associated with activation of the GlyR (12, 13). Similar evidence of conformational change of the M2–M3 domain has been found for the GABA_{A}R (14). We sought to identify structures within the extracellular domain of the human GlyR that are involved in the conformational change that conveys the ligand binding event to opening the channel pore.

The amino acid sequence of the acetylcholine-binding protein (AChBP) from the snail Lymnaea stagnalis, was found to have a strong (15–24%) homology with the extracellular domains of LGIC subunits (15). The crystal structure of this protein provides a template with which to model these extracellular domains (15). Loop 7 of the AChBP corresponds with the conserved Cys-loop of LGICs, which is essential for a functional
The gating of the GlyR. activation (19, 20), resulting in an exaggerated startle phenotype of mice (19, 20). The effect of this mutation is to impair the GlyR corresponds to the location of the A52S mutation in according to the alignment of Brejc (15). Pentameric residues 1 extracellular domain sequence are labeled as N and C, respectively. The figure was generated with Molscript (24).

![A ribbon diagram of the modeled GlyR extracellular domain. The diagram shows the model of the GlyR extracellular domain from outside of the pentameric ring, perpendicular to the 5-fold axis. The side chains of residues Glu-53 and Asp-57 in loop 2 and of Lys-143 and Asp-148 in loop 7 are labeled. The N and C termini of the extracellular domain sequence are labeled as N and C, respectively. The figure was generated with Molscript (24).](image)

**Fig. 1.** A ribbon diagram of the modeled GlyR extracellular domain. The diagram shows the model of the GlyR extracellular domain from outside of the pentameric ring, perpendicular to the 5-fold axis. The side chains of residues Glu-53 and Asp-57 in loop 2 and of Lys-143 and Asp-148 in loop 7 are labeled. The N and C termini of the extracellular domain sequence are labeled as N and C, respectively. The figure was generated with Molscript (24).

**TABLE I**

| GlyR α1      | I_max | EC_{50} | n_{H} | n |
|--------------|-------|---------|-------|---|
| C41A         | 9.24 ± 1.5 | 0.027 ± 0.004 | 2.75 ± 0.3 | 5 |
| E53C         | 2.47 ± 1.9 | 0.90 ± 0.08 | 1.27 ± 0.12 | 4 |
| D57C         | 0.022 ± 0.01 | 1.15 ± 0.24 | 0.78 ± 0.11 | 4 |
| K143C        | 0.94 ± 0.9 | 0.28 ± 0.05 | 1.98 ± 0.24 | 4 |
| D148C        | 0.012 ± 0.003 | 0.97 ± 0.03 | 0.88 ± 0.02 | 4 |

* All values are presented as mean ± S.E.

**EXPERIMENTAL PROCEDURES**

**Molecular Modeling—**Amino acid residues 31–248 of the α1-subunit of the human GlyR (Swiss Prot accession P23415) were aligned with residues 1–206 of the AChBP (Protein Data Bank accession 1I9B) according to the alignment of Brejc (15). Pentameric α1-subunit models of the GlyR were built in a single run of the program Modeler (22), and receptor but not involved in ligand binding (16, 17). The location of loop 7 in the AChBP suggests that the conserved cysteine-loop of LGIC receptors is in a position to interact with the transmembrane domains of the receptor, and so may be involved in gating (15, 18). Loop 2 is also a flexible loop, which in a LGIC receptor subunit is proposed to be in a position to interact with the transmembrane domains. Alignment of the AChBP and GlyR α1-subunit sequences shows that loop 2 corresponds to the location of the A52S mutation in *spasmodic* mice (19, 20). The effect of this mutation is to impair the GlyR activation (19, 20), resulting in an exaggerated startle phenotype, which suggests that loop 2 might also be involved in gating of the GlyR.

As residues and conformational changes in the M2–M3 linker are associated with the activation of the ion channel, we sought to examine the role of charged residues in loops 2 and 7 in the extracellular domain of the GlyR α1-subunit. Using a combination of cysteine accessibility techniques and mutations that reversed the charge of these residues, we evaluated the role of loops 2 and 7 in the activation of the GlyR. We demonstrate that residues in these loops play an important role in receptor activation but not by a simple electrostatic interaction as has been observed in the GABAAR (21).

**Fig. 2.** The effects of MTS reagents on C41A and cysteine-substituted α1 homomeric GlyRs. For the C41A receptor and each of the cysteine-substituted receptors E53C, D57C, K143C, and D148C, the mean response to a maximum concentration of glycine (closed circles) and an approximate EC_{50} concentration (open circles) are shown, normalized to the maximum response prior to any addition of MTS reagents (indicated by –). The results obtained following MTSET reaction (+) with the C41A (n = 3) and MTSES reaction (+) with the C41A (n = 3), E53C (n = 3), D57C (n = 4), and D148C (n = 3) receptor showed no shift in the EC_{50} response. Only the K143C receptor showed any change, with an increase in the EC_{50} response following reaction with MTSET. All values are means ± S.E.

**MTSET and MTSES inactivation** of C41A receptor and each of the cysteine-substituted receptors E53C, D57C, K143C, and D148C, the mean response to a maximum concentration of glycine (closed circles) and an approximate EC_{50} concentration (open circles) are shown, normalized to the maximum response prior to any addition of MTS reagents (indicated by –). The results obtained following MTSET reaction (+) with the C41A (n = 3) and MTSES reaction (+) with the C41A (n = 3), E53C (n = 3), D57C (n = 4), and D148C (n = 3) receptor showed no shift in the EC_{50} response. Only the K143C receptor showed any change, with an increase in the EC_{50} response following reaction with MTSET. All values are means ± S.E.
of 1 min between successive applications. Inhibition curves were constructed from the peak current response to the co-application of a range of taurine concentrations and a fixed concentration of glycine, with a minimum of 1 min between successive applications.

**Fig. 3. The rate of reaction of MTSET with the K143C GlyR.** In panel A, an example of an experiment to determine the rate of reaction in the closed state is shown. Whole cell currents were recorded in response to application of an EC50 concentration of glycine (indicated by open bars), alternating with those recorded during repeated applications of 100 μM MTSET in the absence of glycine (filled bars). The responses show an increase in amplitude during the time course of the reaction. In panel B, the first two whole cell currents are recorded in response to a maximum concentration of glycine (open bars) and an EC50 concentration of glycine (open bars), respectively. These are followed by responses to a maximum concentration of glycine co-applied with 100 μM MTSET (filled and open bars), alternating with test responses to an EC50 concentration of glycine (open bars). This shows the time course of reaction in the open state. The mean data collected in this way for the closed state (filled squares, n = 3) is shown in panel C, with the currents normalized to the response prior to MTSET addition and plotted against cumulative reaction time. Each set of data were fitted with a single exponential. The mean time constants obtained were 1.35 ± 0.25 s for the open states and 5.36 ± 2.5 s for the closed state.

**Table II**

| GlyR a1         | Glycine activation | Taurine activation | Taurine inhibition |
|-----------------|--------------------|--------------------|-------------------|
|                 | I_{max}            | EC_{50}            | n_H               | n       |
|                 |                    |                    |                   |         |
| WT              | 2.87 ± 0.90        | 0.025 ± 0.04       | 1.70 ± 0.14       | 6       |
| E53K            | 0.27 ± 0.09        | 1.17 ± 0.17*       | 1.1 ± 0.03*       | 4       |
| E53D            | 5.29 ± 1.47        | 0.021 ± 0.003      | 1.95 ± 0.12       | 4       |
| D57K            | 0.43 ± 0.16        | 0.23 ± 0.03        | 1.1 ± 0.05*       | 6       |
| D57E            | 4.74 ± 1.88        | 0.008 ± 0.001      | 1.80 ± 0.15       | 4       |
| K143E           | 7.05 ± 1.17        | 0.028 ± 0.002      | 2.02 ± 0.25       | 5       |
| K143R           | 5.51 ± 0.94        | 0.007 ± 0.002      | 2.42 ± 0.11       | 4       |
| D148K           | 0.01 ± 0.002       | 0.96 ± 0.11b       | 0.94 ± 0.07b      | 5       |
| K276E           | 1.1 ± 0.7          | 1.82 ± 0.06b       | 1.50 ± 0.09       | 4       |
|                 |                    |                    |                   |         |
|                 |                    |                    |                   |         |
|                 |                    |                    |                   |         |

* p < 0.01 compared to wild type (WT).
* p < 0.05 compared to wild-type.
* Data reproduced from Lynch et al. (12).

**Relative ΔI_{50} = 1 – ((I_{g0}(t) – I_{g0}(0))/I_{max})** (Eq. 1)

where I_{g0}(t) is the current response to an EC_{50} concentration of glycine before MTS addition, I_{g0}(0) is the current response for each cumulative time point, t, of the MTS reaction and I_{max} is the maximum current response. The data expressed in this way were fitted with a single exponential decay (Origin, Microcal Software, Northampton, MA) to obtain an estimate of the first order rate constant. The averaged first order rate constant was determined from at least 3 cells in each case.

**RESULTS**

**Modeling of the GlyR on the AChBP—** By threading the extracellular domain of the GlyR sequence on the AChBP crystal structure we were able to identify those residues of the GlyR that are located within loop 2 and loop 7, which may be located close to the M2-M3 linker or the ion channel itself (Fig. 1). Amino acid residues with a charged side chain that were located in these loops were chosen for study, namely Glu-53 and Asp-57 in loop 2, and Lys-143 and Asp-148 in loop 7. The residues known to be involved in ligand binding are located distant (25–40 Å) from this site in the receptor (15).

**Effects of Mutations to Cysteine—** All of the cysteine mutants were constructed on an α1-subunit C41A GlyR background to eliminate the possibility of MTS reagents reacting with the free

Sulfhydryl Reagents and Reactions—We used the charged methanesulfonate (MTS) derivatives, 2-sulfonatoethyl methanethiosulfonate (MTSES), and 2-(trimethylammonium)methanethiosulfonate (MTSET) (Toronto Research Chemicals Inc.). Stock solutions of 100 mM MTSES and MTSET in distilled water were aliquoted into screw cap microcentrifuge tubes and rapidly frozen in an ethanol/dry ice mix before storage at –20 °C. For each application of MTS reagents, a new aliquot was thawed, diluted in bathing solution to the working concentration and used immediately.

The rate of covalent modification of cysteine substituted GlyRs by MTS reagents was measured in the presence and absence of glycine. The peak amplitude of the current in response to an EC_{50} concentration of glycine (I_{g0}) and a maximum concentration of glycine (I_{max}) were recorded at least twice prior to MTS reagent addition. For reactions in the open state, 100 μM MTSET or 100 μM MTSES was co-applied with 2 mM glycine. The change in response to an EC_{50} concentration of glycine (relative ΔI_{50}) was expressed relative to the maximum response according to Equation 1,

\[ I = I_{\text{max}}([A]_n[I][A]_{1/2} + EC_{50}^2) \] (Eq. 2)

where I is the peak whole cell current recorded following application of a range of concentrations of the agonist, [A]; I_{max} is the estimated maximum current, EC_{50} is the glycine concentration required for a half-maximum response and n_{H} is the Hill co-efficient.

Statistics were performed on wild-type and mutant EC_{50} values and Hill coefficients using a one-way ANOVA with Fisher’s post-hoc test.
cysteine. This receptor showed no alteration in the glycine sensitivity (EC50 = 0.027 ± 0.004 mM, Table I) or any change after 500 μM MTSES or 500 μM MTSET were added for 1 min (Fig. 2).

To assess the effect of replacing a charged residue with a cysteine, concentration response curves to glycine were measured for the mutant receptors E53C and D57C in loop 2, and K143C and D148C in loop 7. Each of these receptors showed an increase in EC50 with K143C showing the smallest shift (EC50 = 0.28 ± 0.05 mM, 10-fold increase) and D148C showing the largest shift (EC50 = 0.97 ± 0.03 mM, 36-fold increase), compared with the C41A receptor (Table I).

**Effects of MTS Addition to Substituted Cysteine Mutants—**

The covalent modification of cysteine residues enables the introduction of the positive charged trimethyl ammonium group from MTSET or the negative charged sulfonate group from MTSES. For each of the substituted cysteine mutants, this provides a mechanism for reintroducing a charged side chain similar to that of the original charged amino acid. However, this depends upon the substituted cysteine being available for modification. By reacting the positive charged MTSET to the K143C GlyR and the negative charged MTSES to the E53C, D57C, and D148C GlyRs, we could determine whether these cysteine residues were accessible to MTS reagents, as inferred from any detectable shifts in the concentration response curve. When 500 μM MTSES was applied for 1 min to the E53C, D57C, or D148C in the presence or absence of glycine, there was no alteration in the amplitude of the I50 or Imax (Fig. 2). This suggests either the cysteine was not accessible to MTSES for modification, or the sulfonate group of the MTSES had no effect on the response of the mutant receptors.

When 500 μM MTSET was applied to the K143C in the presence or absence of glycine, there was no change in the Imax but a shift in the I50 such that it was now ~80% of the Imax (Fig. 2). A complete concentration-response curve showed that following covalent modification the EC50 had shifted from 242 μM to a value of 70.3 μM, which is closer to that of the C41A GlyR. This indicates that the K143C receptor was accessible for modification by MTSET and the addition of a positive charge partially restored the function of the receptor.

**Rates of Reaction for K143C GlyR—**

To determine if there were conformational changes at the Lys-143 residue between the open and closed states of the receptor, the rate of MTSET modification was measured in both states. For the open state (Fig. 3), the response at an EC50 concentration of glycine (I50) and a maximum concentration of glycine (Imax) were measured before MTSET addition. Then, 100 μM MTSET was applied in the presence of a saturating concentration of glycine (2 mM) for 2 s with at least a 2 min washout before retesting of the I50 and Imax responses. Subsequent applications of 100 μM MTSET in the presence of 2 mM glycine for 3 s, followed by retesting of the
Channel Gating in the Glycine Receptor

Effects of Charge Reversal Mutations—We created the reverse charge mutations E53K and D57K in loop 2, and K143E and D148K in loop 7. In comparison to the wild-type, the E53K GlyR had a 46-fold increase in EC$_{50}$ (0.028 ± 0.003 mM) activation when compared with the wild-type GlyR. Application of taurine to the E53K and D57K GlyRs and the obtained IC$_{50}$ and $I_{\text{max}}$ responses were repeated until the reaction was complete. For the closed state (Fig. 3), the $I_{50}$ and $I_{\text{max}}$ responses were measured after each 3 s application of 100 µM MTSET in the absence of glycine, until the reaction was complete. The change in $I_{50}$ currents were observed relative to the $I_{\text{max}}$ (relative Δ$I_{50}$) and plotted against the cumulative reaction time (Fig. 3C). First order rate constants were estimated from the fit of a single exponential decay to the data. The rate of reaction in the open state ($\tau = 3.56 \pm 2.5$ s, $n = 4$) was faster than that of the closed state ($\tau = 1.35 \pm 0.39$ s, $n = 3$) was faster than that of the closed state of the GlyR. This suggests that the K143C residue is more accessible in the open state than the closed state of the GlyR.

### Table III

| GlyR $a$ | $I_{\text{max}}$ | EC$_{50}$ | $n_H$ | $n$ |
|---------|-----------------|---------|------|-----|
| WT      | 2.87 ± 0.90     | 0.025 ± 0.001 | 1.70 ± 0.14 | 6 |
| E53KK276E | 0.046 ± 0.02    | 1.34 ± 0.09$^b$ | 1.05 ± 0.07$^b$ | 3 |
| D57KK276E | 0.011 ± 0.002   | 0.95 ± 0.05$^b$ | 0.77 ± 0.05$^b$ | 3 |
| D148KK276E | 0.015 ± 0.005  | 1.20 ± 0.13$^b$ | 0.88 ± 0.04$^b$ | 4 |
| E53KK143E | 0.027 ± 0.02    | 1.36 ± 0.08$^b$ | 0.81 ± 0.04$^b$ | 3 |
| D57KK143E | 0.58 ± 0.4      | 0.41 ± 0.02    | 0.98 ± 0.05$^b$ | 5 |

$^a$ All values are presented as mean ± S.E.  
$^b$ p < 0.01 compared to wild-type (WT).

Effects of Double Charge Reversal Mutations—Charged residues located in the M2–M3 linker have previously been implicated in channel gating (12, 13) and this study has demonstrated the role of loop 2 and loop 7 charged residues in gating. Therefore, we tested for electrostatic interactions between the negatively charged residues of loops 2 and 7, and the positively charged Lys-276 residue of the M2-M3 linker by creating the double reverse charge mutations E53KK143E and D57KK143E. The EC$_{50}$ of the E53KK143E mutant was similar to the E53K receptor, and the D57KK143E receptor had shifted slightly to the right of the D57K receptor (Fig. 6; Table III). Therefore, electrostatic interactions between loops 2 and 7 of the extracellular domain do not appear to influence gating.

DISCUSSION

Modeling of the GlyR—By threading the amino acid sequence of the GlyR $\alpha$1-subunit extracellular domain over the AChBP structure, we were able to identify the location of residues that may be involved in various functions of the receptor. In this model, the spasmodic loop and the conserved Cys-loop of the GlyR correspond to loop 2 and loop 7, respectively, of the AChBP structure. These are located in regions that are postulated to be close to the ion channel (15, 18) and to the M2–M3 linker (Fig. 1). We propose that conformational changes occur in the spasmodic loop and Cys-loop of the GlyR upon ligand binding that are an important part of the process linking ligand binding to channel gating. This is consistent with the previous demonstration that the spasmodic mutation results in impaired receptor gating but does not affect ligand binding (19, 20), and the M2–M3 linker is involved in channel gating (12–14).

Conformational Changes That Mediate Channel Activation following Ligand Binding—By using MTS modification of cysteine residues, it is possible to identify residues that are either altered in their own conformation, or else the environment around them is altered by the conformational changes of the receptor. The cysteine-substituted mutant K143C was able to be modified by MTSET, resulting in a decrease in the EC$_{50}$ value. By showing that the rate of this reaction was faster in the open state than the closed state, we demonstrate that this residue is more accessible to modification in the open state. As accessibility is dependent upon the environment surrounding the sulfur atom of the cysteine, these results suggest that the introduced cysteine is in a more hydrophilic environment when the receptor is in the open state compared with the conformation adopted in the closed state (23).

For each of the E53C, D57C, and D148C GlyR mutations, the receptor did not show any change in glycine activation following the addition of MTSET reagents. This could be the result of one of several possibilities. The structure of the receptor may have been altered by the cysteine residue such that the introduced cysteine is not in the same position as the original residues and is now in a hydrophobic environment; the residues may be buried in a pocket that the MTS reagent cannot access, despite actually being surrounded by a hydrophilic en-
environments; or the MTS reagents may react but not change the function of the receptor. We consider the latter possibility unlikely, considering that removal of charge on these residues changes so drastically the function of the receptor. Finally, the residues may shift from a hydrophobic to a hydrophilic area during the signal transduction process, but the length of time that the residues are accessible for modification is so short, because of the altered response of the receptor to glycine, that the MTS reactions are not detectable.

Role of Charged Residues in Loops 2 and 7 of the Extracellular Domain—Both the E53K and D57K mutations resulted in an increased EC$_{50}$ and the agonist taurine was converted to an antagonist. We infer from these results that these two loop 2 charged residues (Glu-53 and Asp-57) are involved in channel gating or the signal transduction process leading to channel gating. The conversion of taurine from an agonist to a partial agonist or an antagonist has previously been demonstrated by mutations within the M2–M3 linker, establishing this region as being involved in channel gating (8). The two charged residues in loop 7 (Lys-143 and Asp-148) were also shown to be involved in channel gating. The D148K mutation, similar to E53K, resulted in an increased EC$_{50}$ and the conversion of the agonist taurine to an antagonist. While the K143E receptor was not different to the wild-type receptor, the K143C mutation did increase the EC$_{50}$. As MTSET reacted faster in the open state, we can infer this residue is not involved in ligand binding, as the MTSET would otherwise be competing with glycine to react at the introduced cysteine and thus would be expected to react faster in the closed state. This is the first study to show the role of charged residues in both loop 2 and loop 7 of the extracellular domain of the GlyR in channel gating.

The reverse charge E53K mutation shifts the concentration-response curve (46-fold increase) to a similar degree as the E53C mutation (33-fold increase). This is not the case for the Asp-57 residue, where the D57K GlyR has a smaller shift in EC$_{50}$ (9-fold increase) compared with the D57C GlyR (42-fold increase). The Lys-143 residue is similarly intriguing, with virtually no change in the EC$_{50}$ for K143E GlyR, but a 10-fold shift in the EC$_{50}$ for the K143C GlyR, compared with wild type. When the loop 7 sequence is aligned with that of other LGICs (Fig. 7), the Lys-143 residue aligns with a positively charged arginine residue in the nAChR $\alpha$7-subunit, however it is a negatively charged glutamic acid residue in the GABA$_{A}$R $\alpha$1-subunit. Therefore, at position 143 of the GlyR the presence of a charge rather than the valency of the charge may be important for the function of the receptor. In comparison, the negatively charged glutamic acid residue at position 53 appears to be conserved as an acidic residue across $\alpha$-subunits, as is the aspartic acid residue at position 148 (Fig. 7).

Charged Residues in Loops 2 and 7 of the GABA$_{A}$R—During the course of this study, it was shown that charged residues in loops 2 and 7 of the GABA$_{A}$R $\alpha$1-subunit interact with residues in the M2–M3 loop to activate channel gating (21). Kash et al. (21) show that three negatively charged residues (Asp-57, Glu-59, and Asp-149) in these loops cause an increase in the EC$_{50}$.

**Fig. 6. Whole cell concentration response curves for single and double charge reversal mutant GlyRs for Lys-143.** In panel A, a comparison is made between the whole cell concentration response curves of the wild-type GlyR (open squares, dashed line) and mutant receptors designed to test for possible electrostatic interactions between the Lys-143 residue in loop 7 and Glu-53 in loop 2. Both the single mutant E53K (filled squares) and the double mutant E53K:K143E (filled circles) exhibited curves with EC$_{50}$ values that were shifted ~50-fold to the right of both the wild-type and K143E (open circles, dashed line) curves. In panel B, the mutations were designed to test for possible electrostatic interactions between the Lys-143 residue in loop 7 and Asp-57 in loop 2. The concentration response curves obtained for the single mutant D57K (filled squares) and double mutant D57K:K143E (filled circles) were shifted to the right of the wild-type (open squares, dashed line) and K143E (open circles, dashed line) curves. The solid and dashed lines in each case indicate the fit to the data obtained with the Hill equation, and the parameters of each fit are shown in Table II for the single mutations and Table III for the double mutations.

**Fig. 7. Sequence alignment of loops 2 and 7 of the extracellular domain of selected subunits of LGIC receptors.** The human sequences for the LGIC subunits shown were aligned to that of the human GlyR $\alpha$1-subunit. Those residues that were investigated in this study (Glu-53, Asp-57, Lys-143, and Asp-148) are shown in circles. The location of the spasmodyc mutation (A52S) in loop 2 is shown in bold. The charged residues in the GABA$_{A}$R $\alpha$1-subunit that were investigated by Kash et al. (21) are boxed. Conserved residues are marked with an asterisk, including the cysteines that form a disulfide bond in the Cys-loop (loop 7).
for GABA when the charge is reversed. A sequence alignment of the GlyR α1- and GABAγR α1-subunits indicates that the residues in the GABAγR α1-subunit involved in gating interactions align with several of the residues chosen for study in the GlyR (Fig. 7). Using mutant cycle analysis of double reverse charge mutations, Kash et al. (21) were able to demonstrate direct electrostatic interactions between both residues D57 (loop 2) and Asp-149 (loop 7) and the Lys-279 residue in the M2–M3 linker of the GABAγR α1-subunit. However, double reverse charge mutations of the Glu-53, Asp-57, and Asp-148 residues and the Lys-143 and Lys-276 residues of the GlyR failed to show any evidence for direct electrostatic interactions. This suggests that while charged residues are clearly involved in the gating process they are not acting through direct electrostatic interactions between these particular pairs of residues in the GlyR.

Implications for Members of the LGIC Receptor Superfamily—This study has confirmed the importance of charged residues in loops 2 and 7 of the extracellular domain of the GlyR in the signal transduction process that links the ligand binding event to channel opening. Unlike the GABAγR (21), there is at present no evidence for direct electrostatic interactions between charged residues in loops 2 or 7 and residue Lys-276 in the M2–M3 loop of the GlyR α1-subunit. This indicates that while the mechanism of signal transduction may be similar between the various receptors of the LGIC superfamily, it is not identical. Studies on the nAChR and 5-HT3 receptor, in addition to further studies on the GABAγR and GlyR, will be necessary to determine the breadth of difference in this mechanism between LGIC receptors. The process of forming theoretical models, mutation and covalent modification analysis and further refining of molecular models will continue to be an invaluable tool in the structural and functional definition of the processes of receptor activation in the LGIC superfamily.