Activation and Desensitization Induce Distinct Conformational Changes at the Extracellular-Transmembrane Domain Interface of the Glycine Receptor*

Qian Wang1 and Joseph W. Lynch2

From the Queensland Brain Institute and School of Biomedical Sciences, University of Queensland, Brisbane QLD 4072, Australia

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Most ligand-gated channels exhibit desensitization, which is the progressive fading of ionic currents in the prolonged presence of agonist. This process involves conformational changes that close the channel despite continued agonist binding. Despite the physiological and pathological importance of desensitization, little is known about the conformational changes that underlie this process in any Cys-loop ion channel receptor. Here we employed voltage clamp fluorometry to identify conformational changes that occur with a similar time course as the current desensitization rate in both slow- and fast-desensitizing α1 glycine receptor chloride channels. Voltage clamp fluorometry provides a direct indication of conformational changes that occur in the immediate vicinity of residues labeled with environmentally sensitive fluorophores. We compared the rates of current desensitization and fluorescence changes at nine labeled extracellular sites in both wild type slow-desensitizing and mutated (A248L) fast-desensitizing glycine receptors. As labels attached to three sites at the interface between the ligand binding domain and transmembrane domain reported fluorescence responses that changed in parallel with the current desensitization rate, we concluded that they experienced local conformational changes associated with desensitization. These labeled sites included A52C in loop 2, Q219C in the pre-M1 domain, and M227C in the M1 domain. Activation and desensitization were accompanied by physically distinct conformational changes at each labeled site. Because activation is mediated by a specific reorganization of molecular interactions at the extracellular-transmembrane domain interface, we propose that desensitization is mediated by a distinct set of conformational changes that prevents this reorganization from occurring, thereby favoring channel closure.

**Background:** Despite its physiological significance, the structural basis of desensitization in Cys-loop receptors is unknown.

**Results:** Fluorescent reporters attached to residues at the interface of the glycine receptor ligand binding and transmembrane domains indicate that activation and desensitization induce distinct local conformational changes.

**Conclusion:** Desensitization induces an interfacial conformational change incompatible with activation.

**Significance:** This provides a mechanism for understanding desensitization.

Glycine receptor chloride channels (GlyRs)3 belong to the Cys-loop family of ligand-gated ion channel receptors that also includes the nicotinic acetylcholine receptor (nAChR), the GABA_A receptor, and the 5-HT_4 (serotonin type-3) receptor. Synaptic GlyRs mediate inhibitory neurotransmission in the spinal cord, brainstem, and retina although non-synaptic GlyRs are widely distributed in neurons throughout the brain and are also present in non-neuronal cells such as leukocytes, macrophages, and sperm cells (1).

Cys-loop receptors comprise pentameric assemblies of similar or dissimilar subunits arranged symmetrically around a central ion-conducting pore. Individual subunits comprise a large ligand-binding domain (LBD) and a transmembrane domain (TMD), the latter consisting of a bundle of four α-helices. The LBD consists of a 10-strand β-sandwich made of an inner β-sheet with six strands and an outer β-sheet with four strands (2). The ligand-binding site is situated at the interface of adjacent subunits and is formed by loops A–C from one subunit and loops D–F from the neighboring subunit (2). Agonists binding to these sites induce local conformational changes that are propagated to the distant activation gate formed by the second transmembrane (M2) pore-lining domains. Recent structural and functional studies have generated an abundance of information about the molecular rearrangements mediating channel opening (3–7).

Ligand-gated channels also display a phenomenon termed desensitization, which is the progressive fading of the ionic flux in the prolonged presence of agonist. The rates of onset and recovery from desensitization are important parameters governing the size and decay rate of synaptic currents (8, 9). As one example of the pathological importance of this mechanism, human hereditary mutations that change the rates of onset and recovery from desensitization in GABA_A receptors and nAChRs have been shown to be associated with different forms of epilepsy (10, 11). Despite the physiological and pathological importance of desensitization in Cys-loop receptors, little is known about the conformational changes that underlie this process.
known about the conformational changes that mediate this process.

According to allosteric receptor theory, ligands stabilize the states for which they display the highest affinity (12). Thus, receptor activation occurs because agonists exhibit a higher affinity for the activated (open) than for the resting (closed) state. Similarly, desensitization occurs because agonists exhibit a higher affinity for the desensitized state than for the activated (open) state. If desensitization alters ligand affinity, then this process must involve a change in structure at the binding site. In addition, desensitization also involves a conformational change that closes the channel. Indeed, the structural basis of this closing mechanism has been elucidated in members of the structurally distinct ionotropic glutamate receptor family (13–16). Although the M2 domains of the nAChR are known to adopt a different closed state structure in the desensitized state relative to the resting closed state (17, 18), there is as yet no model to describe how the structure of the LBD changes during desensitization in any Cys-loop receptor. Site-directed mutations in the LBD do have effects on receptor desensitization rates (10, 19–26), supporting the idea that desensitization-specific conformational changes may occur in this region. However, the fact that mutant receptors exhibit altered desensitization rates “does not necessarily prove that the mutated sites are structural motifs responsible for this process” (8). Although Bouzat et al. (21) employed a chimeric approach to delimit the LDB-TMD interfacial region as a major determinant of Cys-loop receptor desensitization, a more direct method is required to determine whether desensitization-specific conformational changes do indeed occur in this or any other region of the LBD.

In the present study we employed voltage clamp fluorometry (VCF) in an attempt to systematically map LBD conformational changes that move with a similar time course to the desensitization rate as an initial step toward developing a structural model for this mechanism. VCF correlates ion flux rates with conformational changes occurring in real-time in receptor domains of interest (27, 28). It takes advantage of the fact that changes in the quantum efficiency of certain fluorophores occur in response to changes in their immediate chemical microenvironment. Homomeric α1 GlyRs are a useful model system to study this mechanism for two reasons. First, wild type (WT) α1 GlyRs desensitize slowly (29, 30), and site-directed mutations can be introduced at defined intracellular sites to dramatically enhance their desensitization rate (29, 31). Second, we have previously identified numerous residues throughout the LBD, first transmembrane (M1) domain, and external M2-M3 domains of α1 GlyRs that each respond with a robust glycine-induced fluorescence change (ΔF) after labeling with environmentally sensitive fluorophores (32–34). Here we sought to compare time-dependent fluorescent changes from labels attached to 14 LBD and TMD sites in the absence of fast desensitization with those observed at the same labeled sites after an intracellular mutation had been introduced to dramatically enhance the desensitization rate. This enabled us to distinguish those conformational changes that remain unchanged throughout the period of ligand binding from those that tracked the desensitization rate. Although we found no evidence for conformational changes associated with desensitization at any of the labeled sites near the glycine-binding site, we did identify desensitization-specific conformational changes in loop 2 and the pre-M1 domain, which are both structural components of the LBD-TMD interface.

**EXPERIMENTAL PROCEDURES**

**Reagents Used in VCF Experiments—**Sulforhodamine methanethiosulfonate (MTS-R) and 2-((5(6)-tetramethylrhodamine)carboxyamino)ethyl methanethiosulfonate (MTS-TAMRA) were purchased from Toronto Research Chemicals (North York, Ontario, Canada). Alexa Fluor 546 C3 maleimide (AF546) and tetramethylrhodamine-6-maleimide were purchased from Invitrogen. MTS-R, MTS-TAMRA, and tetramethylrhodamine-6-maleimide were dissolved in dimethyl sulfoxide (DMSO) and stored at −20 °C. AF546 was dissolved directly into ND96 solution (96 mM NaCl, 2 mM KCl, 1 mM MgCl2, 1.8 mM CaCl2, 5 mM HEPES) on the day of the experiment and stored on ice for up to 6 h. Glycine stocks were dissolved in ND96 and stored at −20 °C.

**Molecular Biology—**The rat α1 GlyR subunit cDNA was subcloned into the pGEMHE expression vector. The WT and all mutant constructs incorporated the C41A mutation to eliminate the sole uncross-linked extracellular cysteine. QuikChange (Stratagene, La Jolla, CA) was used to generate all cysteine mutants used in this study. The successful incorporation of the mutations was confirmed by the automated sequencing of the entire coding sequence. Capped mRNA for oocyte injection was generated using mMessage mMachine (Ambion, Austin, TX).

**Oocyte Preparation, Injection, and Labeling—**Oocytes from *Xenopus laevis* (Xenopus Express) were prepared as described previously (32) and injected with 10 ng of mRNA. The oocytes were then incubated at 18 °C for 3–7 days in a solution containing 96 mM NaCl, 2 mM KCl, 1 mM MgCl2, 1.8 mM CaCl2, 5 mM HEPES, 0.6 mM theophylline, 2.5 mM pyruvic acid, 50 μg/ml gentamycin (Cambrex Corp., East Rutherford, NJ), pH 7.4. On the day of recording, oocytes were transferred into ND96 containing 10–20 μM concentrations of fluorophore. Typical labeling times were 30 s for MTS-R and MTS-TAMRA (on ice), 30 min for tetramethylrhodamine-6-maleimide (on ice), or 45 min for AF546 (at room temperature). Oocytes were then washed thoroughly and stored in ND96 on ice for up to 6 h before recording. All fluorophores employed here respond with an increase in quantum efficiency as the hydrophobicity of their environment is increased (27, 33). Each cysteine mutant was incubated with all four fluorophores in turn, and generally the one yielding the largest glycine-induced ΔF was analyzed. As unmutated GlyRs never exhibited a ΔF or a change in electrophysiological properties after fluorophore incubation, we rule the possibility of labels binding nonspecifically to receptors.

**VCF and Data Analysis—**The experimental set up comprised an inverted fluorescence microscope (IX51, Olympus, Tokyo, Japan) equipped with a high-Q tetramethylrhodamine isothiocyanate filter set (Chroma Technology, Rockingham, VT), a LUCPlanFLN 40×/NA0.6 objective (Olympus), and a Photomax 200 photodiode (Dagan Corp., Minneapolis, MN) with a 12 V/100 watt halogen lamp (Olympus) as light source. The recording chamber was similar to those described previously.
TABLE 1
Summary of results for glycine-activated current and fluorescence responses
Electrophysiological and fluorescence results are shown in normal and bold type, respectively.

<table>
<thead>
<tr>
<th>Construct</th>
<th>EC$_{50}^\text{M}$</th>
<th>$n_H$</th>
<th>$\Delta I_{\text{max}}^a$</th>
<th>$\Delta F_{\text{max}}$</th>
<th>$n$</th>
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<tbody>
<tr>
<td>A248L α1 GlyR unlabeled</td>
<td>310 ± 30</td>
<td>2.1 ± 0.2</td>
<td>8.3 ± 1.1</td>
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<tr>
<td>A52C/A248L α1 GlyR unlabeled</td>
<td>368 ± 39</td>
<td>2.5 ± 0.2</td>
<td>6.7 ± 0.9</td>
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<tr>
<td>A52C/A248L α1 GlyR MTS-TAMRA</td>
<td>422 ± 20</td>
<td>2.0 ± 0.1$^b$</td>
<td>5.9 ± 0.6</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>A52C/A248L α1 GlyR MTS-TAMRA</td>
<td>1395 ± 145$^b$</td>
<td>1.7 ± 0.2</td>
<td>6.4 ± 0.8</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Q67C/A248L α1 GlyR MTS-TAMRA</td>
<td>4900 ± 400$^b$</td>
<td>1.5 ± 0.1$^b$</td>
<td>6.1 ± 0.7</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Q67C/A248L α1 GlyR MTS-TAMRA</td>
<td>10300 ± 900$^{b,c}$</td>
<td>1.2 ± 0.1$^{b,c}$</td>
<td>31 ± 3</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>K143C α1 GlyR unlabeled</td>
<td>2969 ± 93$^a$</td>
<td>1.5 ± 0.1$^a$</td>
<td>0.9 ± 0.1$^a$</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>K143C α1 GlyR MTS-R</td>
<td>2372 ± 313$^a$</td>
<td>1.0 ± 0.1$^b$</td>
<td>1.8 ± 0.2$^b$</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>K143C α1 GlyR MTS-R</td>
<td>748 ± 97$^{a,b}$</td>
<td>0.8 ± 0.1$^{a,b}$</td>
<td>0.6 ± 0.1</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>K143C/A248L α1 GlyR unlabeled</td>
<td>2970 ± 93$^a$</td>
<td>1.5 ± 0.1</td>
<td>0.9 ± 0.1</td>
<td>3</td>
<td></td>
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<tr>
<td>K143C/A248L α1 GlyR MTS-R</td>
<td>2372 ± 313</td>
<td>1.0 ± 0.1</td>
<td>1.8 ± 0.2</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>K143C/A248L α1 GlyR MTS-R</td>
<td>748 ± 97$^b$</td>
<td>0.8 ± 0.1</td>
<td>0.6 ± 0.1</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>G181C/A248L α1 GlyR MTS-R</td>
<td>1086 ± 99$^b$</td>
<td>1.8 ± 0.03</td>
<td>1.7 ± 0.5$^a$</td>
<td>4</td>
<td></td>
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<tr>
<td>G181C/A248L α1 GlyR MTS-R</td>
<td>5400 ± 400$^{a,b}$</td>
<td>1.1 ± 0.04$^{a,b}$</td>
<td>2.7 ± 0.2$^a$</td>
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<tr>
<td>G181C/A248L α1 GlyR MTS-R</td>
<td>1700 ± 200$^{a,b}$</td>
<td>1.2 ± 0.07$^{a,b}$</td>
<td>1.1 ± 0.3</td>
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<tr>
<td>H201C/A248L α1 GlyR unlabeled</td>
<td>1304 ± 108</td>
<td>1.8 ± 0.1</td>
<td>5.3 ± 0.3$^a$</td>
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<td>H201C/A248L α1 GlyR MTS-R</td>
<td>960 ± 75$^a$</td>
<td>1.6 ± 0.1</td>
<td>7.2 ± 0.4$^a$</td>
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<td>H201C/A248L α1 GlyR MTS-TAMRA</td>
<td>361 ± 44$^a$</td>
<td>1.2 ± 0.1$^a$</td>
<td>2.1 ± 0.07</td>
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<tr>
<td>N203C/A248L α1 GlyR unlabeled</td>
<td>1050 ± 74</td>
<td>1.5 ± 0.1$^a$</td>
<td>7.8 ± 0.6</td>
<td>3</td>
<td></td>
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<tr>
<td>N203C/A248L α1 GlyR MTS-TAMRA</td>
<td>2403 ± 410</td>
<td>2.2 ± 0.1</td>
<td>6.8 ± 0.6</td>
<td>3</td>
<td></td>
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<tr>
<td>N203C/A248L α1 GlyR MTS-TAMRA</td>
<td>682 ± 61$^a$</td>
<td>1.1 ± 0.03$^{a,b}$</td>
<td>9.2 ± 0.3</td>
<td>4</td>
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<tr>
<td>Q219C/A248L α1 GlyR MTS-R</td>
<td>997 ± 136$^a$</td>
<td>1.6 ± 0.1</td>
<td>7.9 ± 1.4</td>
<td>4</td>
<td></td>
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<tr>
<td>Q219C/A248L α1 GlyR MTS-R</td>
<td>508 ± 39$^b$</td>
<td>1.2 ± 0.1$^b$</td>
<td>5.3 ± 0.9</td>
<td>4</td>
<td></td>
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<tr>
<td>Q219C/A248L α1 GlyR MTS-R</td>
<td>214 ± 37$^b$</td>
<td>1.0 ± 0.1$^{a,b}$</td>
<td>3.8 ± 0.4</td>
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<td>Q219C/A248L α1 GlyR MTS-R</td>
<td>53 ± 0.8$^b$</td>
<td>1.5 ± 0.1$^b$</td>
<td>1.9 ± 0.3$^b$</td>
<td>4</td>
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<tr>
<td>Q219C/A248L α1 GlyR MTS-R</td>
<td>25 ± 2$^{a,b}$</td>
<td>1.3 ± 0.1$^b$</td>
<td>1.5 ± 0.3$^b$</td>
<td>4</td>
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<tr>
<td>G221C/A248L α1 GlyR MTS-R</td>
<td>887 ± 64$^a$</td>
<td>2.4 ± 0.1</td>
<td>8.3 ± 1.1</td>
<td>4</td>
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<tr>
<td>M227C/A248L α1 GlyR unlabeled</td>
<td>382 ± 106$^b$</td>
<td>1.4 ± 0.1$^b$</td>
<td>6.0 ± 0.8</td>
<td>4</td>
<td></td>
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<tr>
<td>M227C/A248L α1 GlyR AF546</td>
<td>807 ± 116$^b$</td>
<td>1.4 ± 0.1$^b$</td>
<td>6.0 ± 0.8</td>
<td>4</td>
<td></td>
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<tr>
<td>M227C/A248L α1 GlyR AF546</td>
<td>382 ± 106$^b$</td>
<td>1.4 ± 0.1$^b$</td>
<td>6.0 ± 0.8</td>
<td>4</td>
<td></td>
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</table>

$^a$ Significant difference to electrophysiological properties of unlabeled A248L mutant GlyRs (Student’s t test, $p < 0.05$).
$^b$ Significant difference to electrophysiological properties before labeling in the same mutant GlyR (Student’s t test, $p < 0.05$).
$^c$ Significant difference of fluorescence properties to electrophysiological properties after labeling in the same mutant GlyR (Student’s t test, $p < 0.05$).

Cells were voltage-clamped at −40 mV, and currents were recorded with an OC-725C oocyte amplifier (Warner, Hamden, CT). Current and fluorescence traces were acquired at 200 Hz via a Digidata 1322A interface using pClamp 9.2 software (Axon Instruments, Union City, CA). Fluorescence signals were digitally filtered at 1–2 Hz with an eight-pole Bessel filter and Hill coefficient ($n_H$) values for ligand-induced activation of current and fluorescence were obtained using the Hill equation fitted with a non-linear least squares algorithm (SigmaPlot 9.0, Systat Software, Point Richmond, CA). All results are expressed as the mean ± S.E. of three or more independent experiments. Unless otherwise indicated, statistical analysis was performed using unpaired Student’s t test, with $p < 0.05$ representing significance.

RESULTS

WT α1 GlyR currents exhibit a low rate of desensitization with a $\Delta I$ time constant ($\tau_{\Delta I}$) that typically exceeds 25 s (29). However, the A248L mutation in the intracellular M1-M2 loop increases $\tau_{\Delta I}$ to around 1 s (29). We have previously identified a range of cysteine-substituted residues in the LBD and pre-M1 domains of α1 GlyRs that, when tagged with an environmentally sensitive fluorophore, elicit robust glycine-induced $\Delta F$ responses (32–34). Here we sought to compare the time course of the $\Delta I$ and $\Delta F$ responses of each of these cysteine-substituted receptors with and without the desensitization-enhancing A248L mutation. We reasoned that fluorescence signals that increase or decrease in magnitude with a $\Delta F$ time constant ($\tau_{\Delta F}$) comparable with $\tau_{\Delta I}$ are likely to be reporting a conformational change associated with desensitization. Alternately, those $\Delta F$ signals that remain constant during desensitization are more likely to be reporting a conformational change associated with agonist-binding or receptor activation.

Mean glycine EC$_{50}$, $n_H$, and $\Delta I_{\text{max}}$, $\Delta F_{\text{max}}$ values for the A248L mutant GlyR are summarized in Table 1. We found the glycine EC$_{50}$ value was increased by around an order of magnitude relative to the WT value. We also found the $\Delta I_{\text{max}}$ decayed rapidly and almost completely, with a $\tau_{\Delta I}$ of 1.53 ± 0.13 s ($n = 4$).

We then investigated the following mutations located in either agonist binding domains or extracellular agonist transduction domains: H201C and N203C in binding domain loop C, Q67C in binding domain loop D, S121C and L127C in binding domain loop E, G181C and G221C in binding domain loop F, E217C, Q219C, G221C in the pre-M1 domain, M227C in the M1 domain, A52C in loop 2, K143C in the conserved Cys-loop (or loop 7), and R271C in the M2-M3 domain. Unfortunately, four of the double mutant receptors were not suitable for investigation. The S121C/A248L and L127C/A248L mutant GlyRs exhibited extremely low glycine sensitivities such that 300 mM glycine activated non-desensitizing currents, the E217C/A248L mutant GlyR did not produce a detectable $\Delta F$ with any of the four tested fluorophores, and the R271C/A248L mutant GlyR did not express at all. The remaining nine mutant GlyRs all exhibited robust glycine-activated $\Delta I$ and $\Delta F$ responses when expressed either as single mutants or as double mutants with
A248L. The $\tau_{\Delta I}$ values for all double mutant GlyRs in response to a saturating ($EC_{200}$) glycine are comparable with the value observed at the A248L mutant GlyR (Table 2).

The standard experimental approach applied to all cysteine-substituted GlyRs is illustrated in the example for the G181C mutant GlyR in Fig. 1. This mutation is located in loop F, which lines the base of the glycine binding pocket. First, we quantified the $\Delta I$ and $\Delta F$ concentration-response relationships in the MTS-R-labeled G181C/A248L GlyR. A single concentration-response is shown in Fig. 1A, and averaged results are plotted in Fig. 1B. The mean glycine $EC_{50}$ and $n_H$ values of best fit to individual concentration-response relationships are summarized in Table 1. The corresponding values for the MTS-R-labeled G181C GlyR have been published previously (33). In the labeled double mutant, the glycine $EC_{50}$ value (5.4 ± 0.4 mM) is significantly higher ($p < 0.05$) than the corresponding $\Delta F$ $EC_{50}$ value (1.7 ± 0.2 mM). The corresponding values in the single mutant were reversed, with mean values of 39.8 ± 1.8 μM for $\Delta I$ and 508 ± 65 μM for $\Delta F$ (33). It is evident from Fig. 1A that $\Delta I$ desensitized rapidly, whereas $\Delta F$ did not. This is particularly evident when responses are compared with those of the single mutant MTS-R-labeled G181C GlyR at a saturating glycine concentration (Fig. 1C). To compare the decay rates of the $\Delta I$ and $\Delta F$ signals in both mutants, we expressed averaged signal magnitudes at 20 s after commencement of the glycine application as a percentage of their respective maximum values. We then normalized the averaged $\Delta I/\Delta I_{\text{max}}$ and $\Delta F/\Delta F_{\text{max}}$ data for the G181C GlyR to a value of one and applied the same normalization factor to the respective responses observed at the G181C/A248L GlyR. The averaged results shown in Fig. 1D confirm that the $\Delta I/\Delta I_{\text{max}}$ ratio declined significantly in the double mutant GlyR, whereas the $\Delta F/\Delta F_{\text{max}}$ ratio did not. We thus conclude the fluorophore covalently attached to G181C does not detect a conformational change associated with desensitization.

The Q67C, H201C, N203C, and G221C mutant GlyRs all responded in a similar manner to the G181C mutant GlyR. The fluorescent labels employed at each receptor are the same as used previously (33) and are listed in Table 1. Sample $\Delta I_{\text{max}}$ and $\Delta F_{\text{max}}$ responses for each labeled single- and double-mutated

### Table 2

<table>
<thead>
<tr>
<th>Construct</th>
<th>$\tau_{\Delta I}$ (ms)</th>
<th>$\tau_{\Delta F}$ (ms)</th>
<th>$n_H$</th>
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<tr>
<td>A248L</td>
<td>1532 ± 128</td>
<td>NA</td>
<td>4</td>
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<tr>
<td>A52C/A248L</td>
<td>3497 ± 200</td>
<td>3123 ± 362</td>
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<td>Q67C/A248L</td>
<td>2855 ± 87</td>
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<td>K143C/A248L</td>
<td>2380 ± 351</td>
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<td>G181C/A248L</td>
<td>903 ± 78</td>
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<td>G221C/A248L</td>
<td>1015 ± 32</td>
<td>ND</td>
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<td>H201C/A248L</td>
<td>1276 ± 153</td>
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<td>N203C/A248L</td>
<td>2634 ± 192</td>
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<td>Q219C/A248L</td>
<td>1960 ± 292</td>
<td>2995 ± 243</td>
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<tr>
<td>M227C/A248L</td>
<td>2320 ± 129</td>
<td>2185 ± 158</td>
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### Figures

**Figure 1. Current and fluorescence responses recorded from MTS-R-labeled G181C and G181C/A248L mutant GlyRs.** A, shown are corresponding current and fluorescence traces, activated by the indicated concentrations of glycine and recorded from an oocyte-expressing labeled G181C/A248L mutant GlyRs. In this and Figs. 2–5, black traces denote current recordings, red traces denote fluorescence recordings, and black horizontal bars denote the duration of application of glycine. B, shown are averaged glycine $\Delta I$ and $\Delta F$ concentration-response relationships at labeled G181C/A248L mutant GlyRs. Parameters of best fit to individual concentration-response relationships are given in Table 1. C, shown are examples of $\Delta I_{\text{max}}$ and $\Delta F_{\text{max}}$ responses in labeled G181C and G181C/A248L mutant GlyRs. Note the rapid desensitization of the double mutant GlyR. D, shown are current and fluorescence signal magnitudes recorded 20 s after the commencement of glycine application were normalized to their respective peak values for both GlyRs. The mean percentage $\Delta I/\Delta I_{\text{max}}$ and $\Delta F/\Delta F_{\text{max}}$ values for the G181C GlyR were averaged and normalized to one. The same normalization factor was applied to the respective responses observed at the G181C/A248L GlyR. $***, p < 0.001$ by unpaired t test.
Glycine Receptor Desensitization

![Diagram of glycine receptor desensitization](http://www.jbc.org/)

**FIGURE 2. Current and fluorescence responses recorded from labeled mutant GlyRs that showed no evidence of desensitizing ΔF responses.** A, shown are corresponding current and fluorescence traces, activated by indicated concentrations of glycine and recorded from oocytes expressing the indicated mutant GlyRs labeled as listed in Table 1. Dashed lines indicate the steady-state fluorescence levels observed in the absence of glycine. The 2% ΔF scale bar applies to the two right-most fluorescence traces, whereas the 20% ΔF scale bar applies to the other six. B, shown are the mean percentage ΔI/ΔI_{max} and ΔF/ΔF_{max} values, quantitated 20 s after initial glycine application for the single and double mutant GlyRs. Data were normalized as described in Fig. 1D. ***p < 0.001 by unpaired t test.

receptor are shown in Fig. 2A with averaged 20 s ΔI/ΔI_{max} and ΔF/ΔF_{max} values shown in Fig. 2B. It is evident that ΔF does not report a conformational change associated with desensitization in any of these mutants. The ΔI_{max} and ΔF_{max} values together with the glycine EC_{50} and n_{H} values of best fit to ΔI and ΔF concentration-response curves for all displayed double mutant constructs are summarized in Table 1, whereas the corresponding values for the single mutant constructs have previously been published (33). Together, the results presented in Figs. 1 and 2 indicate that we were not able to detect conformational changes associated with desensitization at any tested location near the glycine-binding site.

Ala-52 is located in loop 2, a domain that has long been implicated in conformational changes associated with receptor opening (3–7). The MTS-TAMRA-labeled A52C mutant GlyR elicits a robust glycine-induced ΔF that is thought to report a conformational change associated with the transition from the resting closed state to the glycine-bound pre-open (or flip) state (34). Sample ΔF_{max} and ΔI_{max} responses from MTS-TAMRA-labeled A52C and A52C/A248L mutant GlyRs are shown in Fig. 3A. The τΔI and τΔF values at the double mutant receptor were both significantly reduced relative to those observed at the single mutant receptor but were not significantly different from each other (Fig. 3B). The glycine concentration-response at the A52C/A248L mutant GlyRs revealed a complex ΔF waveform (Fig. 3C). At low (0.1–0.3 mM) glycine concentrations, there is little evidence of desensitization in either the ΔI or ΔF responses. At glycine concentrations 1 mM, the decay rate of both ΔI and ΔF became progressively faster as glycine concentration was increased. A third feature, apparent only at desensitizing glycine concentrations, is the appearance of a transient increase in ΔF upon glycine removal. The averaged concentration-response relationships for the “glycine-on” peak, the plateau, and the “glycine-off” peak confirm these trends (Fig. 3D). It was not possible to reliably generate ΔF EC_{50} values from these results. Given the result of Fig. 3B, we quantitated the τΔI and τΔF values for glycine concentrations 1 mM by fitting the initial (i.e. glycine-on) decay phase with a single exponential. The results, plotted in Fig. 3E, confirm that there is no significant difference between average τΔI and τΔF values at any glycine concentration, consistent with the ΔF response reporting a conformational change associated with desensitization.

We next sought to determine whether the decay phase of the glycine-off ΔF response may report a conformational change associated with recovery from desensitization. As a first step, we quantitated the ΔI recovery from desensitization time course by applying paired 5-s applications of saturating (30 mM) glycine separated by increasing time intervals. The averaged results, summarized in Fig. 3F, exhibited a half-recovery time of 9.4 ± 1.7 s (n = 5). The averaged half-decay time of the glycine-off ΔF signal, averaged from 6 cells at 30 mM glycine, was 10.9 ± 3.2 s. These values are not significantly different (p = 0.70 by unpaired t test), strongly suggesting that the glycine-off ΔF response at the A52C/A248L mutant GlyR reported a conformational change associated with recovery from desensitization.

Met-227 is located in the M1 domain. We have previously shown that the AF546-labeled M227C mutant GlyR exhibits a glycine-induced ΔF decrease (33). A comparison of the effects of a saturating glycine concentration on ΔI_{max} and ΔF_{max} in the AF546-labeled M227C and M227C/A248L mutant GlyRs is
shown in Fig. 4A. The complex $\Delta F_{\text{max}}$ waveform in the double mutant receptor is reminiscent of that observed in Fig. 3A. The $\tau \Delta I$ and $\tau \Delta F$ values at the double mutant receptor were both significantly faster than those observed at the single mutant receptor but were not significantly different from each other (Fig. 4B). The sample $\Delta I$ and $\Delta F$ glycine concentration-response relationship for the M227C/A248L mutant GlyR, shown in Fig. 4C, reveals a very similar $\Delta F_{\text{glycine}}$ concentration-response profile as observed for the A52C/A248L mutant GlyR (Fig. 3C). To strengthen the case that this $\Delta F$ reports a conformational change associated with desensitization, we quantitated the $\tau \Delta I$ and $\tau \Delta F$ values for glycine concentrations 1 mM by fitting the initial (glycine-on) decay phase with a single exponential. The results, plotted in Fig. 4D, confirm that there is no significant difference between $\tau \Delta I$ and $\tau \Delta F$ values at any glycine concentration, consistent with the interpretation that an AF546 fluorophore attached to M227C reports a conformational change associated with desensitization. We compared the decay phase of the glycine-off $\Delta F$ response with the $\Delta I$ recovery from desensitization time course as described above for the A52C/A248L mutant GlyR. As shown in Fig. 4E, the $\Delta I$ exhibited a half-recovery time of $10.5 \pm 0.8 \text{s}$ ($n = 4$), whereas the averaged half-decay time of the glycine-off $\Delta F$ signal was $15.5 \pm 1.0 \text{s}$ ($n = 5$). Although these values are significantly different ($p < 0.05$ by unpaired $t$ test), their magnitudes are sufficiently close to each other as to suggest that the glycine-off $\Delta F$ response reports a conformational change associated with recovery from desensitization.

Q219C is located in the pre-M1 domain, which connects the LBD and TMD regions. We previously showed that the MTS-
R-labeled Q219C mutant GlyR exhibits a glycine-induced decrease in ∆F (33). The comparison of ∆I/∆I_{max} and ∆F/∆F_{max} responses in MTS-R-labeled Q219C and Q219C/A248L mutant GlyRs in Fig. 5A shows the expected increase in ∆I in the double mutant receptor. This figure also reveals a slowly developing ∆F in the Q219C GlyR and a more rapidly developing ∆F in the Q219C/A248L GlyR. The averaged 20 s ∆I/∆I_{max} ratios confirmed the expected decrease in ∆I in the double mutant receptor (Fig. 5B). The Q219C mutation itself increases the ∆I from >25 s in the WT GlyR to 8.1 ± 1.0 s (n = 4) in the labeled Q219C mutant GlyR. It appears in Fig. 5A and B, that the onset of the ∆F response parallels the ∆I decay rate in both the single and double mutant receptors. We, therefore, hypothesized that the ∆F signal in both the single and double mutant receptors reports a conformational change associated with desensitization. To test this we quantitated the ∆F rise τ values and ∆I decay τ values at a range of glycine concentrations in the Q219C GlyR. The results, summarized in Fig. 5C, demonstrate that the ∆I and ∆F τ values are indistinguishable from each other at each of the tested glycine concentrations. We then performed the same test on the double mutant GlyR. A sample glycine concentration-response for both ∆I and ∆F is shown in Fig. 5D, with the respective averaged τ values shown in Fig. 5E. The correspondence between ∆F rise τ values and ∆I decay τ values is strong but significantly different at low glycine concentrations.

The averaged ∆I recovery from desensitization time courses for the labeled Q219C and Q219C/A248L GlyRs are shown in Fig. 5F and G, respectively. The labeled Q219C GlyR exhibited a ∆I half-recovery time of 22.7 ± 2.2 s (n = 3), whereas the corresponding ∆F half-decay time was 23.6 ± 1.7 s (n = 6). These results are not significantly different from each other (p = 0.76). Similar results were obtained for the double mutant GlyR, where the mean ∆I and ∆F half-recovery times were 17.9 ± 1.5 s (n = 4) and 14.0 ± 1.3 s (n = 5), respectively. These values are also not significantly different to each other (p =
Together, all these results suggest that the ΔF signals in both the MTS-R-labeled Q219C and Q219C/A248L mutant GlyRs report conformational changes associated with the onset and recovery from desensitization.

We have so far demonstrated conformational changes associated with the onset and recovery from desensitization in the pre-M1, M1, and pre-M2 loop domains. These domains, located at the interface between the LBD and TMD, are important for transmitting the agonist binding signal to the gate. In an attempt to refine our understanding of the desensitization conformational changes occurring in this region, we attempted to label further residues in the loop 2 (from E51C/D56C) but were not successful. Because the conserved Cys-loop interacts closely with both loop 2 and the pre-M1 domains and is also important in the channel activation process (3–7), we then attempted to label cysteine-substituted residues between Pro-140 and Asp-148 in this domain. When the K143C mutant GlyR was labeled with MTS-R, it produced mean glycine-induced I\textsubscript{max} and F\textsubscript{max} values of 1.8 ± 0.2 μA and 1.9 ± 0.1% (both n = 4). The ΔI and ΔF glycine concentration-response parameters of best fit for the unlabeled and MTS-R-labeled K143C mutant GlyRs are summarized in Table 1. The ΔF glycine concentration-response parameters of best fit for the MTS-R-labeled K143C mutant GlyRs are also summarized in Table 1.
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The corresponding parameters for the unlabeled and MTS-R-labeled double mutant K143C/A248L GlyR are also summarized in Table 1. In contrast with the labeled K143C mutant GlyR, the $\Delta F$ EC$_{50}$ value is significantly lower than the corresponding $\Delta F$ EC$_{50}$ value. Although the $\Delta I$ of the double mutant GlyR exhibited the expected rapid desensitization rate (Table 2), the $\Delta F$ response showed no significant change over the 20-s period after glycine application, remaining at $97 \pm 3\%$ ($n = 4$) of original magnitude. Thus, we conclude that the conformational change reported by the label attached to K143C is not related to desensitization. However, this does not necessarily mean that the Cys-loop does not experience a desensitization-specific structural change.

**DISCUSSION**

**General Considerations for Data Interpretation**—Recombinant α1 GlyRs do not desensitize rapidly, and the mutation we introduced to enhance desensitization may have introduced structural changes unlike those occurring during “real” fast desensitization in nAChRs or GABAA receptors. However, the converse experimental approach suffers from a similar limitation. That is, if mutations are introduced to eliminate desensitization and activation are not equal and opposite. If they were, then the net glycine-induced closed-flip state transition (34). In the fast-desensitizing A52C/A248L mutant GlyR, we propose that the net glycine-mediated $\Delta F$ response represents the sum of those $\Delta F$ responses corresponding to activation and desensitization. In particular, we suggest that glycine binding induces a stepwise decrease in $\Delta F$ similar to that observed in the labeled single mutant A52C GlyR and that this component of the $\Delta F$ signal reverses completely when glycine unbinds. When this stepwise signal is subtracted from the net $\Delta F$ waveform, we are left with a $\Delta F$ of opposite polarity that we hypothesize reports a conformational change associated with desensitization only. The phase of this signal has the same $\tau_{\Delta F}$ as the $\tau_{\Delta I}$ desensitization rate, and the off-phase has the same half-recovery time as $\tau_{\Delta I}$.

We further suggest that the net $\Delta F$ signal reported by the label attached to the M227C/A248L mutant GlyR can be interpreted in exactly the same way. It is intriguing that the $\Delta F$ plateau signal (represented by open circles in Fig. 3D) at both A52C/A248L and M227C/A248L mutants tends toward increasingly positive $\Delta F$ values as glycine concentration is increased. This implies that the $\Delta F$ due to desensitization is still increasing at glycine concentrations where the $\Delta F$ due to activation has reached a maximum. This is expected because fast desensitization generally only becomes prominent at high agonist EC values. The non-zero $\Delta F$ plateau level indicates that the conformational changes due to desensitization and activation are not equal and opposite to each other. If they were, then the net $\Delta F$ plateau response should asymptote toward zero at high glycine concentrations when the saturating current desensitized completely. We thus conclude that activation and desensitization are mediated by physically distinct conformational changes in loop 2 and the M1 domain.

**Conformational Changes Reported by a Label Attached to Q219C**—Relative to the WT GlyR, the Q219C mutant GlyR exhibits an enhanced desensitization rate. This is consistent with previous studies showing that the desensitization rate is critically dependent on the identity of corresponding residues in the pre-M1 domain of GlyRs (22) and other Cys-loop receptors (19, 21). We conclude that a label attached to the Q219C mutant GlyR reports a conformational change associated with desensitization because the $\Delta F$ on- and off-rates tracked the $\Delta I$

**Conformational Changes Reported by a Label Attached to A52C and M227C**—Loop 2 is well established as a crucial element of the Cys-loop receptor activation machinery (3–7). We previously found that the $\Delta F_{\text{max}}$ of a label attached to A52C in this domain is correlated with agonist efficacy, suggesting that it reports conformation change associated with the agonist-induced closed-flip state transition (34). In the fast-desensitizing A52C/A248L mutant GlyR, we propose that the net glycine-mediated $\Delta F$ response represents the sum of those $\Delta F$ responses corresponding to activation and desensitization. In particular, we suggest that glycine binding induces a stepwise decrease in $\Delta F$ similar to that observed in the labeled single mutant A52C GlyR and that this component of the $\Delta F$ signal reverses completely when glycine unbinds. When this stepwise signal is subtracted from the net $\Delta F$ waveform, we are left with a $\Delta F$ of opposite polarity that we hypothesize reports a conformational change associated with desensitization only. The on-phase of this signal has the same $\tau_{\Delta F}$ as the $\tau_{\Delta I}$ desensitization rate, and the off-phase has the same half-recovery time as $\tau_{\Delta I}$.

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desensitization and recovery rates, respectively, in both the slow-desensitizing Q219C mutant GlyR and the fast-desensitizing Q219C/A248L mutant GlyR.

A Role for These Conformational Changes in Desensitization—To summarize, we have observed desensitization-specific conformational changes in the vicinity of Ala-52 in loop 2, Glu-219 in the pre-M1 domain, and Met-227 in the outer part of M1. In muscle nAChRs and homomeric ρ1 GABA_A receptors, it has been shown that a direct electrostatic interaction exists in the closed state between loop 2 and pre-M1 domain residues that correspond to Glu-53 and Arg-218 in the α1 GlyR (39–41). In the muscle nAChR it was shown that channel activation perturbed this salt bridge (41), although a follow-up study found that this perturbation was unlikely to play a major role in gating (42). Irrespective of the precise movements and mechanisms involved in activation, these results suggest a close physical association between loop 2 and the pre-M1 domain, which in turn implies that desensitization could simply involve an alteration in their relative orientations. However, it is likely that other interfacial domains are also involved in desensitization conformational changes. An agonist-mediated perturbation at this interface is likely to realign all interfacial domains (i.e., the M2-M3 loop, the pre-M1 domain, and LBD loops 2, 7, and 9), inducing the breakage and reformation of numerous bonds (43), leading ultimately to a reconfiguration of the M2-M3 loop and the opening of the channel. The novel insight provided by the present study is that desensitization involves conformational changes in the interface region that are distinct from those involved in activation or deactivation. Such conformational changes would be expected to prevent the activation-triggering molecular realignments from taking place and would thus maintain the channel in the closed state. Our demonstration of desensitization-specific conformational changes at the interfacial and M1 domains explains the results of a recent chimeric study that implicated loop 2, the conserved Cys-loop, and the outer part of M1 as the main elements responsible for the difference in desensitization rates between slow- and fast-desensitizing Cys-loop receptors (21). They also explain the results of site-directed mutagenesis studies that had previously implicated loop 2 and pre-M1 domain residues as structural determinants of Cys-loop receptor desensitization (19, 22, 26, 44).

Conclusion—We employed VCF to directly demonstrate that GlyR activation and desensitization are accompanied by physically distinct conformational changes at the interface between the LBD and the TMD. Because activation is mediated by a specific reorganization of molecular interactions at this interface, we propose that desensitization is mediated by a distinct set of conformational changes that is incompatible with activation and thereby closes the channel. Thus, the locations of the desensitization-specific conformational changes reported here imply that they mediate, rather than occur in response to, desensitization.

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Activation and Desensitization Induce Distinct Conformational Changes at the Extracellular-Transmembrane Domain Interface of the Glycine Receptor
Qian Wang and Joseph W. Lynch

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