Structural Studies of the Actions of Anesthetic Drugs on the γ-Aminobutyric Acid Type A Receptor

Gustav Akk, Ph.D.,* Joe Henry Steinbach, Ph.D.†

ABSTRACT

The γ-aminobutyric acid type A receptor is the major transmitter-gated inhibitory channel in the central nervous system. The receptor is a target for anesthetics, anticonvulsants, and sedatives whose actions facilitate the flow of chloride ions through the channel and enhance the inhibitory tone in the brain. Both the kinetic and structural aspects of the actions of modulators of the γ-aminobutyric acid type A receptor are of great importance to understanding the molecular mechanisms of general anesthesia. In this review, the structural rearrangements that take place in the γ-aminobutyric acid type A receptor during channel activation and modulation are described, focusing on data obtained using voltage-clamp fluorometry. Voltage-clamp fluorometry entails the binding of an environmentally sensitive fluorophore molecule to a site of interest in the receptor, and measurement of changes in the fluorescence signal resulting from activation- or modulation-elicted structural changes. Detailed investigations can provide a map of structural changes that underlie or accompany the functional effects of modulators.

* Assistant Professor of Anesthesiology, Department of Anesthesiology, Washington University School of Medicine, St. Louis, Missouri. † Russell and Mary Shelden Professor of Anesthesiology, Department of Anesthesiology, Washington University School of Medicine.

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Address correspondence to Dr. Akk: Department of Anesthesiology, Washington University School of Medicine, Campus Box 8054, 660 S. Euclid Avenue, St. Louis, Missouri 63110, akk@morpheus.wustl.edu. Information on purchasing reprints may be found at www.anesthesiology.org or on the masthead page at the beginning of this issue. ANESTHESIOLOGY’s articles are made freely accessible to all readers, for personal use only, 6 months from the cover date of the issue.

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Organization and the Static Structure of the γ-Aminobutyric Acid Type A Receptor

The γ-aminobutyric acid type A (GABA_A) receptor constitutes the predominant fast inhibitory force in the central nervous system. The receptor responds to the binding of two synthetically released or ambient gamma-aminobutyric acid (GABA) molecules with a conformational change in the associated channel, opening the channel gate for chloride and other monovalent anions. In mature neurons, the intracellular chloride concentration is low and the chloride reversal potential is negative to the threshold for action potentials. Thus, GABA_A receptor activation results in an influx of anions, leading to hyperpolarization of the cell or reduction of the effects of excitatory channels.

The receptor is a target for numerous anesthetics, anticonvulsants, anxiolytics, and sedatives, which act by facilitating chloride flow through the channel.1–3 Kinetic studies of ionic currents have shown that the drugs act by enhancing the open probability of the channel by stabilizing the open state of the channel and/or destabilizing the closed state. It has been proposed that the drugs act to enhance affinity to GABA (e.g., benzodiazepines4) or gating efficacy (e.g., neurosteroids5), although the precise molecular mechanisms of action are not fully understood.

The GABA_A receptor is a pentameric membrane protein. To date, 19 GABA_A receptor subunits have been cloned. These are: α1–α6, β1–β3, γ1–γ3, δ, ε, π, θ, and ρ1–ρ3.6 Native receptors are heteromeric, typically containing two α subunits, two β subunits, and a single γ, δ, ε, π, or θ subunit. The previously called GABA type C receptors are homooligomers and heterooligomers, formed of ρ1, ρ2, or ρ3 subunits. The expression of the subunits is regionally and developmentally regulated, so that receptors with differing subunit compositions and differing physiologic and pharmacologic properties are expressed throughout the nervous system. The effects of subunit composition on channel properties have been thoroughly described in publications spanning the past two decades.7–11

Each of the five homologous subunits has a large N-terminal extracellular domain. This is followed by four membrane-spanning regions and a short C-terminal tail extending to the outside of the cell12 (fig. 1). A mature subunit is
Channel Activation

The binding of agonist to the extracellular domain of the receptor leads to conformational changes that result in the opening of the channel gate, some 60 Å away. In the related nicotinic acetylcholine receptor, the binding of transmitter leads to movement in loop C, which caps the binding pocket and is believed to constitute the physical rearrangement that initiates propagation of structural changes to the channel gate and underlies increased affinity to the transmitter of the open channel. Similar rearrangements likely take place in the GABA<sub>α</sub> receptor.

Recent studies have introduced voltage-clamp fluorometry (or site-specific fluorescence), an approach in which fluorescence changes, resulting from changes in the microenvironment surrounding a fluorophore upon channel activation, are used to study changes in receptor structure. The fluorophore is an environmentally-sensitive probe that responds with altered quantum yield; that is, altered fluorescence intensity during constant illumination to changes in the polarity of the environment. The probe is covalently attached to a cysteine residue engineered in the site of interest in the receptor. If the environment around the probe changes during channel activation or modulation, the intensity of the fluorescence signal changes. The change in fluorescence intensity will be denoted as \( \Delta F \) in this article. It is usually expressed relative to the baseline signal, so \( \Delta F = (\text{fluorescence signal in test condition}) - (\text{fluorescence signal in control condition}) / (\text{fluorescence in control}) \).

When coupled with electrophysiologic recordings this approach allows simultaneous recording of functional (current flow) and the associated or underlying structural (fluorescence) responses from the receptor. The advantage of voltage-clamp fluorometry is that it has the potential to reveal
changes in receptor structure (conformation) that are not seen in the membrane current response. One particular example that we will discuss is the action of inhibitory steroids on the ρ1 GABA<sub>A</sub> receptor: in all cases the membrane current is reduced but the associated conformational changes in the receptor differ. 21

This approach was initially used in studies of structural changes associated with gating of potassium channels, 22 but has more recently been used to study structural dynamics in transmitter-gated ion channels such as the nicotinic acetylcholine receptor, 23,24 glycine receptor, 25,26 and the GABA<sub>A</sub> receptor. 21,27–30 In the following sections we review the current state of knowledge on the structural dynamics of the GABA<sub>A</sub> receptor.

**Criteria for Observation of Fluorescence Changes**

Observation of fluorescence changes is dependent on a number of criteria, such as successful labeling of the engineered cysteine residue in the region of interest; changes in the polarity of the environment surrounding the fluorescent probe; and a lack of significant labeling of endogenous cysteines that would increase background fluorescence signal or produce an activation-dependent signal of their own. We will now examine each of these criteria separately.

The maleimide- (e.g., Alexa 546 maleimide, A5m) or methanethiosulfonate (MTS)-based fluorescent probes (e.g., sulfonohodaine methanethiosulfonate) covalently bind to the cysteine residue (fig. 2). Cysteines most likely to be successfully labeled are at the water-accessible surface of the protein where, because of ionization of the side chain, the reaction rate is significantly higher. 31 In addition, cysteine residues located in the hydrophobic intraprotein core may be inaccessible to the probe. The intrinsic reaction rate of MTS derivatives with thiols is on the order of 10<sup>7</sup> M<sup>-1</sup> s<sup>-1</sup>. 32 Although the reaction rates with engineered cysteine residues in proteins can be significantly less depending on the location and availability of the residue, complete modification in the experimental setting is typically achieved in seconds or minutes.

Successful binding of the probe to the receptor can often be determined from changes in functional properties, e.g., a shift in the agonist concentration-response curve after incubation with the fluorophore. In the absence of a functional effect, an activation- or modulation-elicted fluorescence change (ΔF) itself can serve as proof of successful labeling of the receptor. The absence of a fluorescence change is more difficult to interpret. Either lack of labeling or lack of measurable environmental changes around the fluorophore will result in a situation where no ΔF is observed.

It is important to remember that the fluorophore senses the polarity of environment. Thus, the structure of the receptor surrounding the fluorophore may change, but unless this leads to changes in the polarity of the environment, the fluorescence signal does not change. An increase in fluorescence intensity is associated with the environment around the fluorophore becoming more hydrophobic. The magnitude of ΔF, however, does not contain information about the physical distance traveled or the origin of movement, i.e., whether the surrounding residues move relative to the static fluorophore or whether the residue linked to fluorophore moves relative to a static environment. We note that conformational changes altering proximity or spatial relation to a protein side chain with quenching activity (e.g., aromatic residues) could also result in changes in fluorescence intensity.

Fluorescence experiments are conducted on receptors expressed in *Xenopus* oocytes. A schematic of a recording setup that combines fluorescence measurements and two-electrode voltage clamp is shown in figure 2.

Control experiments on cells expressing wild-type (α1β2, α1β2γ2, or ρ1) GABA<sub>A</sub> receptors incubated with A5m or sulfonohodaine MTS do not demonstrate changes in the GABA concentration-response relationship or an activation-dependent ΔF. 27,29,30,33 The extracellular domain of the GABA<sub>A</sub> receptor contains 10 native cysteine residues (two in each subunit of the pentameric receptor). These residues are cross-linked to each other to form the characteristic intrasubunit disulfide bond at the interface between the transmitter-binding extracellular domain and the membrane-spanning region, a necessary precondition for functionality in Cys-loop receptors. 34 On the extracellular side of the membrane, in the linker between the second and third membrane-spanning regions, are five cysteine residues (one per subunit), but these are apparently not labeled by the commonly used fluorophores. Additional cysteine residues are found in the transmembrane segments and in the long cytoplasmic loop between the third and fourth membrane-spanning segments. These are likely inaccessible to the fluorophores that are applied to the extracellular medium.

To date, voltage-clamp fluorometry has focused on the extracellular region of the receptor. It was observed from loop F residues A181 to S185 in the α1 subunit loop C residues Q203, S204, and E208 with numerous fluorophores has not been productive. 29 In the extracellular region of the β2 subunit, ΔF signals have been observed from L125 (loop E) and I180 (loop F) residues. No ΔF was observed from S200, T201, G202, or S203 sites (loop C). In the γ2 subunit, labeling at the S195 site (loop F) produces a fluorescence change during channel activation. However, labeling of neighboring sites (R194, W196, or R197) has been unproductive.

In the homooligomeric ρ1 receptor, Chang and Weiss 27 found that the L166 (loop E, homologous to α1L127 site), S66 (at the subunit-subunit interface), and Y241 sites (loop C) respond to agonist applications with fluorescence change.
In loop F of the α1 subunit, Khatri et al. and Zhang et al. found that sites K210, K211, L216, K217, T218, E220, R221, I222, S223, L224, Q226, F227, and I229 exhibit agonist-induced fluorescence changes. Sites that have been successfully labeled in the extracellular domain of the GABAA receptor are shown in figure 3.

In the second membrane-spanning domain of the β2 subunit, the 24’ residue (numbering starts from the intracellular side and is based on consensus sequence with the related nicotinic receptor; K274) can be successfully labeled. In the α1 subunit, labeling of the N274 site (N20’ residue in M2) produces ΔF in the presence of agonists. The sites from 19’ to 27’ do not exhibit ΔF. In the γ2 subunit, labeling of the P23’ site (P288C), but not 20’ or 24’ sites, results in productive labeling. A full list of sites productively labeled is given in table 1.

Thus, productive labeling has been moderately successful, with less than half of all tested sites demonstrating drug-induced ΔF. It should also be noted that ΔF is sometimes observed with one fluorophore but not another, probably

Fig. 2. Voltage-clamp fluorometry. (A) Structures of two commonly-used fluorophores: Alexa 546 maleimide (Alexa 546) and tetramethylrhodamine maleimide (TMRM). For comparison, structure of the transmitter γ-aminobutyric acid (GABA) is shown next to the fluorophores. (B) The maleimide group of the fluorophore binds to the thiol group of the cysteine residue via a maleimide-thiol reaction to form a carbon-sulfur bond. (C) Schematic of the experimental setup. The receptors are expressed in Xenopus oocytes. The oocyte is placed in a custom-made chamber exposing a portion of the membrane through a 0.8-mm pinhole to the lower compartment. The agonist and modulators are applied through the lower compartment. The oocyte is impaled in the top compartment. Two-electrode voltage-clamp is used to record the currents. The chamber is placed into a Petri dish, and an inverted microscope equipped with the appropriate filters is used to illuminate the oocyte and collect fluorescence.
Voltage-clamp Fluorometry on GABA<sub>A</sub> Receptors

Fig. 3. The extracellular domain of the γ-aminobutyric acid type A (GABA<sub>A</sub>) receptor portraying the side view at the β2-α1 subunit interface. The β subunit is shown in cyan, the α subunit is shown in pink. The residues that respond with fluorescence change (ΔF) to applications of agonist are shown in blue in the β subunit, and in red in the α subunit. The numbering pertains to the rat α1 and β2 subunits. Sites for which homologous residues in the ρ1 receptor demonstrate ΔF are shown in gray. For those residues, the numbering derives from the human ρ1 sequence. The transmitter binds in the intersubunit cavity near the residues Y241 and L127. A summary of data from fluorescence recordings from these sites is given in table 1. In fluorescence recordings the residues are mutated to cysteines, which are then labeled with environmentally sensitive fluorescent probes. For size comparison, a commonly-used fluorophore, Alexa 546 maleimide is shown next to the receptor structure.

resulting from differences in the precise positioning of the bound fluorophore molecule. Overall, it appears that the ρ1 receptor may be more amenable to productive labeling, likely because of its homooligomeric nature resulting in the binding of five molecules of the fluorophore per receptor and a larger fluorescence signal. The magnitude of ΔF is expressed in percent in relation to the baseline fluorescence level. A typical response is an increase or decrease of up to 4%, whereas higher ΔF values are common in the homooligomeric ρ1 receptor.

Movements during Channel Activation by Orthosteric Ligands

Receptors productively labeled with a fluorophore demonstrate a change in fluorescence intensity upon exposure to the transmitter. An increase in fluorescence intensity is associated with the environment surrounding the fluorophore becoming less polar, whereas a decrease in fluorescence indicates that the environment has become more polar. A change in fluorescence does not reveal whether the protein region around the fluorophore moved or the fluorophore itself moved, because of dislocation of the residue to which it is linked. Thus, ΔF is an indication of a general structural change in the region where the fluorophore is located. The sign of ΔF can be important when comparing the actions of different ligands that may cause distinct structural rearrangements. The magnitude of the change is important in that increased signal-to-noise ratio improves signal detection; however, the magnitude of ΔF cannot be used as an indication of the extent or distance of movement. Overall, the approach is most useful when comparing the effects of different drugs to determine whether a structural change has taken place with little regard concerning the nature or extent of movement.

Sites near the transmitter binding site show ligand-induced ΔF. A sample recording is shown in figure 4A. Several important points can be made on the basis of published work. First, the time course of development of the ΔF signal from the GABA<sub>A</sub> receptor is in most cases similar to the time course of current rise. This is an indication that ΔF is associated with channel activation. However, in some cases, a disconnect between the current and ΔF time courses has been noted. In the ρ1K217C (loop F) receptor labeled with sulforhodamine MTS, Zhang et al. observed that the fluorescence signal developed more rapidly than the current response. They proposed that the ΔF from the ρ1K217C receptor reports the agonist binding event rather than channel gating. Overall, however, the solution exchange rates in the oocyte system are too slow to resolve fast steps of channel activation.

During prolonged agonist exposure the GABA<sub>A</sub> receptor desensitizes, which in current recordings manifests as reduced amplitude of the response. The data on the time courses of simultaneous fluorescence traces are scant. For the α1 subunit E122C and L127C sites (both in loop E), two studies found no evidence of a concurrent sag in the ΔF signal, indicating that the fluorescent reporters at the transmitter binding site are not sensing rearrangements associated with channel desensitization.

An increase in applied GABA concentration leads to an increase in the current response and the ΔF. In most instances, the concentration-response relationships are indistinguishable for the two, indicating that the conformational changes underlying the ΔF are associated with channel activity. Akk et al. showed that the fitted EC<sub>50</sub> for current and fluorescence responses from the α1L127Cβ2γ2 receptor labeled with A5m were within a factor of two from each other. In the ρ1 receptor, labeled with A5m at the Y241C (loop C), L166C (loop E), or S66C sites (subunit interface), the GABA-elicited current and ΔF curves are essentially superimposable. In contrast, the ρ1K217C residue has the ΔF concentration-response curve left-shifted by sixfold in comparison with the activation curve. It was proposed that in the ρ1K217C receptor the structural rearrangements take place, and are observed as ΔF, under conditions where only one or two of the five agonist binding sites are occupied, whereas currents can be observed after the binding of a minimum of three GABA molecules.

Higher ΔF signal at higher GABA concentrations indicates a correlation between the conformational changes underlying the fluorescence change and some aspect of channel activation, such as a higher fraction of occupied or active receptors. This implies that partial agonists and/or competi-
### Table 1. Summary of Fluorescence Signals from the GABA<sub>A</sub> Receptor

<table>
<thead>
<tr>
<th>Location</th>
<th>Labeled Residue</th>
<th>Receptor</th>
<th>Activator</th>
<th>Fluorophore</th>
<th>ΔF</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Loop C</td>
<td>ρ1Y241C</td>
<td>ρ1</td>
<td>GABA, 3-APMPA, 3-APA</td>
<td>A5m</td>
<td>↑</td>
<td>21,27</td>
</tr>
<tr>
<td>Loop E</td>
<td>α1E1122C</td>
<td>α1β2</td>
<td>GABA</td>
<td>MA, MTMR</td>
<td>↓</td>
<td>33</td>
</tr>
<tr>
<td>Loop E</td>
<td>α1E1122C</td>
<td>α1β2</td>
<td>GABA</td>
<td>TMRM</td>
<td>↓</td>
<td>33</td>
</tr>
<tr>
<td>Loop E</td>
<td>α1E1122C</td>
<td>α1β2γ2</td>
<td>GABA</td>
<td>MTMR</td>
<td>↓</td>
<td>33</td>
</tr>
<tr>
<td>Loop E</td>
<td>α1L127C</td>
<td>α1β2</td>
<td>GABA</td>
<td>A5m, MTMR</td>
<td>↑</td>
<td>30</td>
</tr>
<tr>
<td>Loop E</td>
<td>α1L127C</td>
<td>α1β2γ2</td>
<td>GABA</td>
<td>A5m, MTMR</td>
<td>↑</td>
<td>30</td>
</tr>
<tr>
<td>Loop E</td>
<td>ρ1L166C</td>
<td>ρ1</td>
<td>GABA, TACA</td>
<td>A5m</td>
<td>↑</td>
<td>21,27</td>
</tr>
<tr>
<td>Loop E</td>
<td>ρ1L166C</td>
<td>ρ1</td>
<td>3-APMPA, 3-APA</td>
<td>A5m</td>
<td>↓</td>
<td>27</td>
</tr>
<tr>
<td>Loop F</td>
<td>α1R186C</td>
<td>α1β2</td>
<td>GABA, β-alanine, gabazine</td>
<td>MTSR</td>
<td>↓</td>
<td>29</td>
</tr>
<tr>
<td>Loop F</td>
<td>β2180C</td>
<td>α1β2</td>
<td>GABA, β-alanine, gabazine</td>
<td>MTSMR</td>
<td>↓</td>
<td>29</td>
</tr>
<tr>
<td>Loop F</td>
<td>γ2S195C</td>
<td>α1β2γ2</td>
<td>GABA, β-alanine</td>
<td>TMRM</td>
<td>↓</td>
<td>33</td>
</tr>
<tr>
<td>Loop F</td>
<td>γ2S195C</td>
<td>α1β2γ2</td>
<td>GABA, β-alanine</td>
<td>TMRM</td>
<td>↓</td>
<td>33</td>
</tr>
<tr>
<td>Loop F</td>
<td>ρ1K210C</td>
<td>ρ1</td>
<td>GABA</td>
<td>MTSR</td>
<td>↑</td>
<td>35</td>
</tr>
<tr>
<td>Loop F</td>
<td>ρ1K211C</td>
<td>ρ1</td>
<td>GABA</td>
<td>MTSR</td>
<td>↑</td>
<td>35</td>
</tr>
<tr>
<td>Loop F</td>
<td>ρ1L216C</td>
<td>ρ1</td>
<td>GABA, isoguvacine, I4AA, 3-APA</td>
<td>A5m</td>
<td>↓</td>
<td>28</td>
</tr>
<tr>
<td>Loop F</td>
<td>ρ1L216C</td>
<td>ρ1</td>
<td>GABA, 3-APMPA</td>
<td>MTSR</td>
<td>↓</td>
<td>35</td>
</tr>
<tr>
<td>Loop F</td>
<td>ρ1K217C</td>
<td>ρ1</td>
<td>GABA, isoguvacine, I4AA, 3-APA, 3-APMPA</td>
<td>A5m</td>
<td>↓</td>
<td>28,35</td>
</tr>
<tr>
<td>Loop F</td>
<td>ρ1T218C</td>
<td>ρ1</td>
<td>GABA, isoguvacine, I4AA, 3-APA</td>
<td>A5m</td>
<td>↑</td>
<td>28</td>
</tr>
<tr>
<td>Loop F</td>
<td>ρ1T218C</td>
<td>ρ1</td>
<td>GABA, 3-APMPA</td>
<td>MTSR</td>
<td>↓</td>
<td>35</td>
</tr>
<tr>
<td>Loop F</td>
<td>ρ1E220C</td>
<td>ρ1</td>
<td>GABA</td>
<td>A5m, MTSR</td>
<td>↑</td>
<td>28,35</td>
</tr>
<tr>
<td>Loop F</td>
<td>ρ1R221C</td>
<td>ρ1</td>
<td>GABA</td>
<td>A5m</td>
<td>↑</td>
<td>28</td>
</tr>
<tr>
<td>Loop F</td>
<td>ρ1R222C</td>
<td>ρ1</td>
<td>GABA</td>
<td>A5m</td>
<td>↑</td>
<td>28</td>
</tr>
<tr>
<td>Loop F</td>
<td>ρ1L222C</td>
<td>ρ1</td>
<td>GABA, 3-APMPA</td>
<td>MTSR</td>
<td>↓</td>
<td>35</td>
</tr>
<tr>
<td>Loop F</td>
<td>ρ1L222C</td>
<td>ρ1</td>
<td>GABA, 3-APMPA</td>
<td>MTSR</td>
<td>↓</td>
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<tr>
<td>Loop F</td>
<td>ρ1S223C</td>
<td>ρ1</td>
<td>GABA, isoguvacine, I4AA, 3-APA</td>
<td>A5m</td>
<td>↓</td>
<td>28</td>
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<tr>
<td>Loop F</td>
<td>ρ1L224C</td>
<td>ρ1</td>
<td>GABA</td>
<td>A5m</td>
<td>↓</td>
<td>28</td>
</tr>
<tr>
<td>Loop F</td>
<td>ρ1Q226C</td>
<td>ρ1</td>
<td>GABA</td>
<td>A5m</td>
<td>↓</td>
<td>28</td>
</tr>
<tr>
<td>Loop F</td>
<td>ρ1F227C</td>
<td>ρ1</td>
<td>GABA</td>
<td>A5m</td>
<td>↓</td>
<td>28</td>
</tr>
<tr>
<td>Loop F</td>
<td>ρ1I229C</td>
<td>ρ1</td>
<td>GABA, isoguvacine, TACA, 3-APA</td>
<td>A5m</td>
<td>↓</td>
<td>28</td>
</tr>
<tr>
<td>ECD</td>
<td>ρ1S66C</td>
<td>ρ1</td>
<td>GABA, TACA</td>
<td>A5m</td>
<td>↓</td>
<td>21,27</td>
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<tr>
<td>ECD</td>
<td>ρ1S66C</td>
<td>ρ1</td>
<td>3-APMPA</td>
<td>A5m</td>
<td>↓</td>
<td>21,27</td>
</tr>
</tbody>
</table>

The table gives a list of residues for which changes in fluorescence signal have been observed. The columns give the labeled residue, its location in receptor, receptor subunit composition, ligands used to activate or modulate the receptor, the fluorophore, a direction of ΔF, and the reference. GABA, β-alanine, and TACA are full agonists of the GABA<sub>A</sub> receptor. Isoguvacine and I4AA are partial agonists considered to act at the transmitter binding site. The receptor by interacting with an allosteric binding site. Gabazine is a competitive inhibitor of the α2 and α4β2 receptor. 3-APMPA and 3-APMPA are competitive inhibitors of the ρ1 receptor. The ρ1L166 site is homologous to the L127 residue in the α1 subunit.

* Activating concentration (300–800 μM) PB; ** 100 μM diazepam; *** 10 μM diazepam.

A5m = Alexa 546 maleimide; 3-APMPA = 3-aminopropyl-(methyl)phosphinic acid; 3-APA = 3-aminopropylphosphonic acid; ECD = extracellular domain; GABA = γ-aminobutyric acid; I4AA = imidazole-4-acetic acid; M2 = second membrane-spanning domain; MTSMR = sulfonohidamine methanethiosulfonate; MTSMR = 2-(5(6)-tetramethylrhodamine)carboxylamino)ethyl methanethiosulfonate; PB = pentobarbital; TACA = trans-aminoacrotic acid; TMRM = tetramethylrhodamine maleimide.

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tive antagonists should exhibit ΔF that is distinct from that for full agonists if the ligand-induced movements are translated to channel gating. However, the experimental support for this is scarce.

In the homooligomeric ρ1 receptor, Khatri et al. showed no correlation between the ability of the ligand to elicit a ΔF and to activate the receptor. The partial agonists imidazole-4-acetic acid and isoguvacine exhibited a narrow range of ΔF from the various mutants, regardless of the level of activation. In the α1L127Cβ2γ2, Akk et al. showed a lack of correlation between the peak current and magnitude of ΔF when the parameters for GABA were compared with those for the partial agonists piperidine-4-sulfonic acid (P4S) or 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol (THIP). The ΔF in the presence of P4S or tetrahydroisoxazolo[5,4-c]pyridin-3-ol was significantly larger than in the presence of GABA. In contrast, β-alanine and muscimol elicited smaller currents and a smaller fluorescence change compared with GABA. Together, these data suggest that the ΔF readings in these examples report local structural changes associated with ligand binding rather than global structural changes associated with transduction of binding to movement of the gate.

Competitive inhibitors interact with the transmitter binding site but do not activate the receptor. Competitive antagonism can thus be considered an extreme case of partial agonism. The ΔF observed in the presence of competitive inhibitors should reflect structural changes that are not associated with channel opening. Indeed, Muroi et al. showed that α1β2γ2 receptors labeled at the α1L127C residue with tetramethylrhodamine maleimide (TMRM) exhibit an increase in fluorescence intensity when the receptor is activated by GABA, but a decrease in fluorescence when the receptor is exposed to the competitive inhibitor gabazine. This suggests that the two ligands produce different structural changes. It was proposed that in the presence of GABA the GABA-binding cavity closes and thereby shortens the distance between the neighboring α and β subunits, a process that may initiate the global rearrangements leading to channel gating. Exposure to gabazine, in this scenario, causes an opposite change in fluorescence that may reflect structural changes associated with competitive inhibition.

Fig. 4. Sample current and fluorescence recordings from the α1L127Cβ2γ2 receptor labeled with A5m. Receptors were expressed in Xenopus oocytes, and studied with two-electrode voltage clamp. (A) Exposure to γ-aminobutyric acid (GABA) elicits an inward current response (I) and an increase in fluorescence intensity (ΔF), suggesting that the environment around the fluorophore becomes more hydrophobic during channel activation. Channel desensitization does not affect fluorescence changes. (B) Exposure to the competitive antagonist gabazine does not activate the receptor but elicits fluorescence change (ΔF). For comparison, responses to GABA from the same cell are also shown. (C) Activation by GABA but not pentobarbital induces ΔF. Both sets of traces are from the same cell. (D) Relationship between current and fluorescence change. The responses are normalized to those to GABA. The normalized ΔF is plotted as a function of normalized current response. P4S = piperidine-4-sulfonic acid; THIP = 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol; 3α5αP = allopregnanolone; ETO = etomidate; β-Ala = β-alanine, PB = pentobarbital. A, C, and D are reproduced with permission from the British Journal of Pharmacology.
pattern of movement that does not lead to channel opening. In contrast, Akk et al.\(^3^0\) found that when α1L127Cβ2γ2 receptors were labeled with A5m, GABA and gabazine elicited ΔF of the same sign (fig. 4B). The two fluorophores, TMRM and A5m, differ in their linker lengths (5 Å for TMRM, 15 Å for A5m), meaning that the fluorophores bound to the same residue are positioned in different locations and report different changes in environment. It is unlikely that the structural rearrangements themselves differ in receptors labeled with different fluorophores.

In the α1β2γ2 receptor labeled with TMRM at the γ2S195C site (loop F), Wang et al.\(^2^9\) found a correlation between the ability of a ligand (GABA, β-alanine, gabazine) to induce currents and ΔF. Loop F of the γ subunit does not contribute to the transmitter binding site, indicating that the allosteric conformational change in loop F of the γ subunit is associated with channel activation. Labeling of loop F residue in the α1 subunit (α1R186C) results in ΔF, which is similar in magnitude and sign for GABA, β-alanine, and the antagonist gabazine.\(^2^9\) This suggests that the ΔF for loop F of the α subunit reflects the binding event. Similarly, loop F residues in the ρ1 receptor show similar ΔF in the presence of GABA and the competitive inhibitors 3-aminopropylphosphonic acid or 3-aminopropyl-(methyl)phosphinic acid.\(^2^8,^3^5\)

In ρ1 receptors labeled at the Y241C residue (loop C), the competitive inhibitor 3-aminopropyl-(methyl)phosphinic acid, like GABA, leads to an increase in fluorescence, although the magnitude of change is smaller than in the presence of the transmitter.\(^2^7\) However, when the receptors are labeled at the L166C (loop E) or S66C residue, 3-aminopropyl-(methyl)phosphinic acid elicits ΔF that has an opposite sign to the ΔF in the presence of GABA. Exposure to the competitive antagonist 3-aminopropylphosphonic acid elicits ΔF of the same sign as GABA when the receptors are labeled at the Y241C residue. In ρ1L166C receptors, the ΔF has an opposite direction to that of GABA, and no ΔF is observed at the S66C site. Overall, the data indicate that the classic competitive antagonists do not act solely to sterically prevent the binding of GABA, and that these drugs can induce structural changes of their own. A full summary of fluorescence changes observed during channel activation is given in table 1.

**Movements during Channel Activation by Allosteric Ligands**

The GABA\(_A\) receptor can be directly activated by many neurosteroids, barbiturates, and general anesthetics such as etomidate and propofol. These drugs interact with sites that are distinct from the transmitter binding site. The binding site mediating direct activation by neurosteroids is located in the membrane-spanning region at the interface between α and β subunits, where it is lined by the αT236 and βY284 residues (numbering from the rat α1 and β2 subunits).\(^1^6\) Etomidate binds to a pocket in the membrane-spanning region at the interface between the α (α1M236) and β (β3M286) subunits.\(^3^7\) The barbiturate binding site is likely located within the membrane-spanning region although the details are currently unknown.

Fluorescence studies have shown that channel activation by these allosteric activators does not typically elicit structural changes at the transmitter binding site. Muroi et al.\(^3^3\) examined the current and fluorescence responses from the α1E122C and α1L127C sites (loop E) and the homologous residues in the β2 subunit. Pentobarbital, at concentrations that elicit direct responses without significant channel block, did not induce changes in fluorescence intensity from TMRM-labeled receptors expressed in αβ or αγ configuration. Work on A5m-labeled α1L127Cβ2γ2 receptors found that fluorescence was not affected during channel activation by saturating concentrations of pentobarbital, etomidate, or the neurosteroid allopregnanolone\(^3^0\) (fig. 4C and D). Clearly, GABA and pentobarbital can activate the receptor without inducing similar structural changes near the transmitter binding site, but it should be noted that both allopregnanolone and etomidate are relatively ineffective activators of the α1L127Cβ2γ2 receptor and that the low activation status may have precluded the observation of the ΔF signal.

Fluorescence elicited from a residue near the top of M2 (24') in the β2 subunit, β2K274C, shows different responses to GABA and pentobarbital.\(^3^7\) In receptors consisting of α and β subunits, pentobarbital elicits an increase in fluorescence whereas GABA is without effect. In αβγ receptors, GABA elicits a negative ΔF, whereas low concentrations of pentobarbital are without effect. These findings indicate that GABA and pentobarbital activate the GABA\(_A\) receptor by mechanisms involving different structural changes in the receptor. A summary of the fluorescence data is presented in table 1.

**Movements during Modulation**

In addition to direct activation, allosteric ligands such as neurosteroids, barbiturates, etomidate, and propofol potentiate GABA\(_A\) receptor activity elicited by low concentrations of GABA.\(^4^6,^4^3\) Benzodiazepines (e.g., diazepam), for which direct activation has not been found, can enhance GABA\(_A\) receptor activity.\(^4^4\)

Several studies have investigated structural changes that take place during positive or negative modulation of the GABA\(_A\) receptor. Wang et al.\(^2^9\) found that a residue in loop F in the γ2 subunit (γ2S2195C) responded with a decrease in fluorescence when the receptor was activated by GABA, whereas fluorescence levels increased when benzodiazepine diazepam and GABA were coapplied. A positive ΔF was also observed with the benzodiazepine inverse agonist, methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate, indicating that the two benzodiazepine site ligands induce local conformational changes associated with occupation of the benzodiazepine site. Diazepam also induces a conformational change in the M2-M3 linker region (N20°C) of the α
subunit. Interestingly, the sign of $\Delta F$ depends on the presence of the $\gamma$ subunit, suggesting that the occupation of the high-affinity benzodiazepine site in $\alpha\beta\gamma$ receptors leads to a structural rearrangement that is different from the change in $\alpha\beta$ receptors containing only the low-affinity site.45

Akk et al.30 examined the potentiating effect of a selected group of modulators on $\alpha1\beta22\gamma2$ receptors labeled with A5m at the $\alpha1L127C$ site. In a simple allosteric model for receptor activation, an open state of the receptor is associated with increased affinity for agonist. This model predicts that a drug that increases channel activation at a given agonist concentration would also increase $\Delta F$ because of increased occupancy of the transmitter binding site. It was found that coapplication of etomidate or the neurosteroid allopregnanolone with a low concentration of GABA led to an increase in the current response and an enhanced fluorescence change. In contrast, the presence of pentobarbital led to an enhanced current response without affecting $\Delta F$. The findings on pentobarbital were confirmed by examining potentiation of currents elicited by a partial agonist, P4S, and during coapplication of pentobarbital and gabazine. Gabazine acts as a noncompetitive antagonist of channel opening by pentobarbital, reducing the peak current by stabilizing the inactive state of the receptor. This experiment allows independent verification of the interactions of both drugs with the receptor: the binding of pentobarbital is confirmed by the presence of the current response, and the binding of gabazine to the same population of receptors is confirmed by the reduction of the current amplitude. In the $\alpha1L127CB22\gamma2$ receptor, gabazine elicits a fluorescence change whereas pentobarbital does not. The coapplication of pentobarbital and gabazine would be expected to shift the receptor population toward states with low affinity to the inhibitor reducing the number of receptors with gabazine bound, and hence reduce the $\Delta F$ from the receptor population. However, the gabazine-induced $\Delta F$ was not affected by pentobarbital. Thus, three separate experiments indicate that the application of pentobarbital does not affect $\Delta F$ at the $\alpha1L127C$ site. Overall, the results can be interpreted in the framework of a model where exposure to etomidate and allopregnanolone, but not pentobarbital, causes structural changes in the transmitter binding site that can be associated with increased affinity to the agonist.30

A study comparing the effects of selected neurosteroids on the $\rho1$ receptor labeled at Y241C (loop C), L166C (loop E), K217C (loop F), or S66C (subunit-subunit interface) found that the steroids, in addition to reducing peak current, could in some instances reduce the magnitude of $\Delta F$.21 The effects on fluorescence were specific to a combination of steroids and sites. For example, the application of sulfated steroids, e.g., pregnanolone sulfate or allopregnanolone sulfate, reduced the $\Delta F$ from receptors labeled at the $\rho1K217$ site. In contrast, the application of pregnanolone, 5$\beta$-tetrahydrodeoxy corticosterone, or 17$\beta$-estradiol was without effect on fluorescence. When the receptors were labeled at the L166C site, 17$\beta$-estradiol, but not pregnanolone, 5$\beta$-tetrahydrodeoxy corticosterone, pregnanolone sulfate, or allopregnanolone sulfate, influenced the magnitude of $\Delta F$. Application of the sulfated steroids and 17$\beta$-estradiol affected the fluorescence change produced at the $\rho1$S66C site. None of the steroids tested affected $\Delta F$ at the $\rho1Y241$ residue. Steroid-induced effects on conformational changes in the extracellular domain are generally not consistent with the steroids acting as classic open-channel blockers. It was concluded that the steroids act through an allosteric mechanism; however, given the distinct conformational changes in the extracellular domain, different steroids can act through different molecular mechanisms.21

Picrotoxin is a noncompetitive antagonist with its interaction site in the channel pore.46 Its mechanism of action is not solely pore block, rather it is allosteric and can be use-dependent.37 The application of picrotoxin alone elicits no $\Delta F$ from residues ($\alpha1E122C$ and $\alpha1L127C$) near the transmitter binding site of the $\alpha1\beta22\gamma2$ receptor.33 In the $\rho1$ receptor, coapplication of picrotoxin with GABA reduces the peak current but has little effect on $\Delta F$ for A5m-labeled Y241C or L166C receptors. When the receptor is labeled at the S66C site the application of picrotoxin reduces, with similar concentration-dependency, the current and fluorescence changes.27 These findings indicate that picrotoxin-mediated antagonism is associated with long-distance structural changes, although the effect on the structure of the transmitter binding site may be limited given the lack of effect on $\Delta F$ produced at the Y241C and L166C sites. In loop F, the presence of picrotoxin reduces $\Delta F$ from the K210C, K217C, T218C, and I222C residues, but is without effect at fluorescence changes from K211C or L216C.35

**Summary of Fluorescence Studies and Comparison with Other Structural Studies**

Voltage-clamp fluorometry can be a powerful tool in the studies of receptor dynamics. Some of the findings have been useful in confirming results from previous studies using other techniques (e.g., the substituted cysteine accessibility method48). Others have provided novel structural information, such as the effect of desensitization on structural rearrangements that may be difficult to obtain with conventional approaches and techniques. A distinctive feature of voltage-clamp fluorometry is that the $\Delta F$ signal reports real-time environmental changes whereas the substituted cysteine accessibility method reports a functional effect because of the binding of the modifying agent. In this respect, both methods complement each other. We now provide a brief comparison of the salient results from fluorescence studies on GABA$\A$ receptors with studies involving the effects of MTS reagents on function.

Drugs considered to act through competitive antagonism can in some instances elicit fluorescence changes. Two aspects of this phenomenon should be considered. First, the fact that a competitive antagonist is capable of eliciting $\Delta F$ suggests that the drug acts not solely by sterically interfering with agonist binding but rather can induce a structural change of its own. These findings and conclusion are in
agreement with previous work in which the substituted cysteine accessibility method was used. Boileau et al. found that gabazine triggers a conformational rearrangement near the β2D95C (loop A) residue. Gabazine also induces a structural change in loop E in the α1 subunit. Second, in some instances the agonist (GABA) and competitive antagonist (gabazine) elicit ΔF that have opposite signs. This can be interpreted as the two classes of drugs inducing distinct structural changes: one type in the presence of an agonist, another in the presence of a competitive antagonist. A previous study found that GABA and gabazine differentially affect the chemical reactivity of MTS reagents with engineered cysteine residues in the transmitter binding site.

The comparison of the pattern of fluorescence signals indicates that GABA and the allosteric activator pentobarbital elicit nonidentical structural changes. This finding is in agreement with results from studies using the substituted cysteine accessibility method. In the transmitter binding site, MTS reactivity is similarly affected by GABA and pentobarbital at the α1E122C site, but differentially affected at the α1L127C site. In the pre-M1 region both GABA and pentobarbital induce structural changes. However, the nature of rearrangements is different. GABA affects the reaction rate for an MTS reagent with the β2K213C residue but is without effect on α1K221C. For pentobarbital the effect is opposite: pentobarbital affects the reaction rate at the α1K221C site but not β2K213C. The differences in structural changes may be lost near or at the channel gate. At the M2 6th level GABA and high, directly-activating concentrations of pentobarbital (as well as general anesthetics propofol and isoflurane) induce similar conformational changes as evidenced by disulfide trapping between engineered cysteines. The single-channel conductance is also indistinguishable for GABA and pentobarbital, indicating that the open channel structures are alike.

Studies on the ρ1 receptor found that inhibitory drugs can interfere with structural changes in the extracellular domain. However, the effects differ for different agents, which produce a pattern of effects falling into one of three distinct classes. These findings are supported by a previous electrophysiologic study that proposed a similar classification of inhibitory agents based on their sensitivity to mutations in the membrane-spanning domain.

We have examined only a few anesthetics using this approach. Because many anesthetics appear to have similar actions to stabilize the open state of the GABA _A_ receptor, additional information on structural consequences of anesthetic action has the potential to separate anesthetics based on mechanism as well as binding site, rather than functional effect.

A critical step is to examine the effects of anesthetic drugs on resting or transmitter-elicited fluorescence changes at additional sites in the receptor. Our current stable of sites is quite restricted and must be expanded.

Overall, voltage-clamp fluorometry has strong potential to clarify the similarities and differences of the mechanisms of action for anesthetic compounds. We are currently faced with the situation that anesthetics enhance GABA _A_ receptor activation, but there is little understanding of the structural basis. Greater insight into the structural basis of potentiation will certainly enhance our understanding of anesthetic mechanisms, and has the potential to guide further explanation of drug structure-activity relations.

**Future Directions**

Fluorescent reporters provide data complementary to functional data on channel activation, and enhance our ability to construct models for understanding drug action. We believe that this approach opens up a number of opportunities for novel insights into anesthetic actions.

One necessary, initial step is to continue to work to associate fluorescence signals with particular states of the receptor (e.g., bound, activated, desensitized or modulated), an area that is currently poorly explored.

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