Functional Consequences of the Loss of High Affinity Agonist Binding to \(\gamma\)-Aminobutyric Acid Type A Receptors

IMPLICATIONS FOR RECEPTOR DESENSITIZATION

J. Glen Newell§§ and Susan M. J. Dunn‡¶

From the §Department of Pharmacology and ¶Centre for Neuroscience, University of Alberta, Edmonton, Alberta T6G 2H7, Canada

We reported previously that tyrosine 62 of the \(\beta2\) subunit of the \(\gamma\)-aminobutyric acid, type A (GABA\(_A\)) receptor is an important determinant of high affinity agonist binding and that recombinant \(\alpha 1\beta2\gamma2\) receptors carrying the Y62S mutation lack measurable high affinity sites for \([^{3}H]\)muscimol. We have now examined the effects of disrupting these sites on the macroscopic desensitization properties of receptors expressed in Xenopus oocytes. Desensitization was measured by the ability of low concentrations of bath-perfused agonist to reduce the current responses elicited by subsequent challenges with saturating concentrations of GABA. Wild-type receptors were desensitized by pre-perfused muscimol with an IC\(_{50}\) \(\approx 0.7\) \(\mu M\), which correlates well with the lower affinity sites for this agonist that are measured in direct binding studies. Receptors carrying the \(\beta2\) Y62S and Y62F mutations desensitized at slightly higher (2-7-fold) agonist concentrations. However, at low perfusate concentrations, the Y62S-containing receptor recovered from the desensitized state even in the continued presence of agonist. The characteristics of desensitization in the wild-type and mutant receptors lead us to suggest that the major role of the high affinity agonist-binding site(s) of the GABA\(_A\) receptor is not to induce desensitization but rather to stabilize the desensitized state once it has been formed.

Desensitization, an intrinsic biophysical characteristic of ligand-gated ion channels, facilitates neurobiological adaptation to prolonged or repeated exposure to agonist. The molecular mechanisms by which this occurs have been subject to intense investigation but remain poorly understood (1). However, the general consensus is that agonist-induced conformational changes of the receptor protein induce the initial, if not all, phases of the process (2, 3).

The \(\gamma\)-aminobutyric acid type A receptors (GABA\(_A\)Rs)\(^3\) are members of a receptor gene family (see Ref. 4) that includes the nicotinic acetylcholine receptors (nACHRs), glycin receptors, and the serotonin type 3 receptor. These receptors are structurally and functionally homologous and desensitize in the continued presence of agonist (5, 6). GABA\(_A\)Rs are large pentameric protein complexes composed of subunit isoforms from a number of classes (\(\alpha 1-\alpha 6\), \(\beta 1-\beta 3\), \(\gamma 1-\gamma 3\), \(\delta\), \(\epsilon\), and \(\pi\)). Although the precise subunit compositions of native receptors remain to be established, a major subtype in the brain appears to be a combination of \(\alpha 1\), \(\beta 2\), and \(\gamma 2\) subunits in a likely stoichiometry of 2:2:1 (7–9).

Radioligand binding studies have revealed the existence of (at least) two classes of agonist recognition sites on a single GABA\(_A\)_R (10), which differ in affinity by more than 1 order of magnitude (see also Refs. 11–13). By using the recombinant rat \(\alpha 1\beta2\gamma2\) receptor expressed in tsA201 cells, we found two classes of binding sites for \([^{3}H]\)muscimol with \(K_d\) values of 8.1 and 430 \(nM\) (10). Because higher concentrations of muscimol are required to activate this receptor subtype (EC\(_{50}\) of 7.1 \(\mu M\) when expressed in Xenopus oocytes), sites of still lower affinity may be present (see below). Although the roles of these multiple sites in receptor function require clarification, it is generally accepted that the high affinity binding that is measured under equilibrium conditions reflects binding to a desensitized conformation(s) of the receptor (see Ref. 14). Furthermore, it is assumed that agonist occupancy of these sites in the resting state of the protein induces the conformational changes that lead to this equilibrium desensitized state.

One approach to delineate the role of individual sites in receptor function is to use site-directed mutagenesis to disrupt selectively binding domains and to examine the consequences on receptor function. By using this approach, we have recently identified an amino acid residue (Tyr-62) of the \(\beta 2\) subunit of the GABA\(_A\)_R that is important for high affinity agonist binding (10). Mutation of this residue to a phenylalanine (Y62F) caused a significant decrease in agonist affinity, and mutation to a serine (Y62S) led to a loss of measurable high affinity binding sites.

In this study, we have expressed recombinant wild-type and mutant GABA\(_A\)_R receptors in Xenopus oocytes and have developed methods to study aspects of the desensitization process. Receptors carrying the Tyr-62 mutations retained the ability to be desensitized upon prolonged exposure to agonists but with slightly altered concentration dependence compared with the wild type. At low GABA concentrations (1–10 \(\mu M\)), the Y62S mutant receptor displayed the unusual property of first becoming desensitized but then recovering from desensitization despite the continued presence of agonist. Thus, under these conditions, this receptor (which lacks measurable high affinity agonist-binding sites) seems to be unable to maintain the de-
sensitized state. Therefore, we propose a new model in which the role of the high affinity sites is not to induce desensitization but rather to stabilize the desensitized state once it has been formed.

**EXPERIMENTAL PROCEDURES**

**Site-directed Mutagenesis—**GAB<sub>A</sub> α2β subunit mutants were engineered using the Altered Sites II<sup>®</sup> in vitro Mutagenesis System (Promega, Madison, WI) as described previously (10, 15).

**cRNA Transcription and Oocyte Preparation—**Xenopus oocytes were prepared as described (16). Capped cRNA transcripts for GAB<sub>A</sub>α2 subunits were prepared from cDNA constructs that were generous gifts from Dr. David Weiss. Oocytes were injected with α1, β2 (or β2 mutants), and γ<sub>2L</sub>-subunit cRNA (1 μg/μl total RNA) in a 1:1:1 ratio in a total volume of 50 nl. Individual oocytes were maintained in 96-well plates at 14 °C in modified Barth’s medium (88 mM NaCl, 1 mM KCl, 0.5 mM CaCl<sub>2</sub>, 0.5 mM Ca(NO<sub>3</sub>)<sub>2</sub>, 2.5 mM sodium pyruvate, 1 mM MgSO<sub>4</sub>, 2.4 mM NaHCO<sub>3</sub>, 15 mM HEPES, pH 7.4) that was supplemented with 100 μg/ml gentamicin.

**Two-electrode Voltage Clamp—**Electrophysiological experiments were performed 2–7 days following oocyte injection. Standard two-electrode voltage clamp techniques were carried out using a GeneClamp 500B Amplifier (Axon Instruments Inc.) at a holding potential of −60 mV. The microelectrodes were filled with 3 M KCl and had resistances of 0.5–5 MΩ. Megahm in frog Ringer’s medium (120 mM NaCl, 1.8 mM CaCl<sub>2</sub>, 2 mM KCl, and 5 mM HEPES, pH 7.4). In all experiments, oocytes were continuously bathed in frog Ringer’s via a gravity perfusion system at a flow rate of 5–7 ml/min. GABA (Sigma) or muscimol (Sigma) was dissolved in the same medium.

**Concentration Dependence of Desensitization—**The current amplitude elicited by a 30-s application of a saturating concentration of GABA (1 mM) was first stabilized prior to experiments such that a maximum variation of 5% was observed over three successive applications. In desensitization experiments, changes in the current amplitudes elicited by challenge applications of GABA (1 mM or muscimol 2 mM, i.e. concentrations that elicited a maximal response in all recombinant receptors, were measured. Bath pre-perfusion with lower concentrations of agonist was started after recovery of receptors from the desensitization induced by the challenge application itself. The relative current amplitude used in determination of IC<sub>50</sub> values for desensitization was defined by the steady state current (I<sub>c</sub>) that was achieved in the presence of the indicated concentration of agonist in the pre-perfusion (see example in Fig. 1). Data were analyzed by iterative curve fitting using Equation 1 (GraphPad Prism Software) as follows:

\[ I = I_c(1 + (IC_{50}/[A])^n) \]  

(Eq. 1)

where I<sub>c</sub> is the peak amplitude of the current elicited by a given concentration of agonist ([A]); I<sub>cmax</sub> is the maximum amplitude of the current; IC<sub>50</sub> is the concentration required for half-maximal inhibition; and n is the Hill coefficient. The normalized data are presented as percent control to construct concentration-response curves.

**Characterization of Desensitization—**The assay protocols were optimized to ensure that any observed desensitization was due only to the presence of agonist in the bathing medium and not to other variables. In experiments to measure the effects of frequency of challenge and duration of pre-perfusion on current amplitude, desensitizing GABA concentrations equivalent to their IC<sub>50</sub> values (determined as above) were used in the pre-perfusion. Where noted in the text, pre-perfusion was started either after washout of the challenge concentration or immediately during the recovery phase from the agonist challenge. Both pre-perfusion protocols gave the same maximum depression of current amplitude under steady state conditions.

**Rate of Recovery from GABA-induced Desensitization—**The rate of recovery from desensitization was measured by first applying a desensitizing concentration of GABA (its E<sub>k</sub>max or EC<sub>50</sub> concentration as noted) and then monitoring the magnitude of the peak current elicited by the same concentration of GABA administered at different time intervals (1–30 min) after the initial challenge. The recovery of the current amplitude as a function of time was fit by a single exponential model (GraphPad Prism, San Diego, CA; www.graphpad.com) to give estimates of the rate and extent of recovery. Similar experiments were carried out in the presence of bath-perfused GABA (3 μM) to assess the effects of pre-perfusion on the recovery from desensitization. Data were fitted to Equation 2,

\[ \text{Recovery} = Y_0 + Y_R(1 - \exp(-k't)) \]  

(Eq. 2)

where Y<sub>0</sub> represents the baseline response to GABA; Y<sub>YR</sub> is recovery; k<sub>r</sub> is the rate of recovery; and t is the time.

**Statistical Analyses—**Data were analyzed by a one-way analysis of variance followed by either a post hoc Dunnett’s test or Newman-Keuls to determine levels of significance. The data for GABA-mediated current shown in Figs. 2 and 3 were analyzed by a one-way repeated measures analysis of variance to compare the current before and after bath pre-perfusion of the indicated concentration of GABA.

**RESULTS**

**Agonist-induced Desensitization**

Fig. 1A shows representative two-electrode voltage clamp recordings from recombinant wild-type α1β2γ2<sub>L</sub> receptors expressed in Xenopus oocytes. The oocyte was challenged at regular time intervals (12 min) using a concentration of agonist that elicited a maximum response (1 mM). Perfusion of low concentrations of GABA in the bath results in a decrease in the maximum amplitude (I<sub>max</sub> or EC<sub>50</sub>) of the induced currents. This inhibition reaches a steady state, and the currents return to control levels after washout of agonist from the perfusate. A, bath pre-perfusion with 10 μM GABA causes an immediate reduction in the current induced by the first challenge; B, perfusion of 1 μM GABA does not immediately desensitize the receptor, but the apparent desensitization becomes pronounced with successive challenge applications.

**Experimental Procedures**

Site-directed Mutagenesis—GABA<sub>A</sub> α2β subunit mutants were engineered using the Altered Sites II<sup>®</sup> in vitro Mutagenesis System (Promega, Madison, WI) as described previously (10, 15).

**cRNA Transcription and Oocyte Preparation—**Xenopus oocytes were prepared as described (16). Capped cRNA transcripts for GABA<sub>A</sub>α2 subunits were prepared from cDNA constructs that were generous gifts from Dr. David Weiss. Oocytes were injected with α1, β2 (or β2 mutants), and γ<sub>2L</sub>-subunit cRNA (1 μg/μl total RNA) in a 1:1:1 ratio in a total volume of 50 nl. Individual oocytes were maintained in 96-well plates at 14 °C in modified Barth’s medium (88 mM NaCl, 1 mM KCl, 0.5 mM CaCl<sub>2</sub>, 0.5 mM Ca(NO<sub>3</sub>)<sub>2</sub>, 2.5 mM sodium pyruvate, 1 mM MgSO<sub>4</sub>, 2.4 mM NaHCO<sub>3</sub>, 15 mM HEPES, pH 7.4) that was supplemented with 100 μg/ml gentamicin.
The effects of the time of pre-perfusion on the current depression were also investigated. By using a 9- or 12-min interval between challenges, the control responses were reproducible in amplitude, and there was no difference in the magnitude of the inhibition observed during agonist perfusion or in the recovery upon washout (Fig. 2A). A 3-min interval is clearly not suitable for measuring the concentration dependence of desensitization, because the receptor is unable to recover from the desensitization induced by the challenge itself. This is shown by the decrease in the amplitudes of successive control currents prior to inclusion of the lower concentration of GABA in the bath. By using a 6-min interval, there were apparent frequency-dependent effects on washout, i.e. the current did not immediately return to control levels. Similar control experiments have been carried out using muscimol (data not shown). In all cases, a 12-min interval between challenges gave rise to reproducible effects in which the apparent desensitization depends only on the presence of agonist in the perfusion medium and on the number (see e.g. Fig. 1B) but not on the frequency of channel activations.

Pre-perfusion Time—The effects of the time of pre-perfusion were also investigated. Fig. 2B shows representative experiments using wild-type receptors. In these experiments, responses to 1 mM GABA were first stabilized; the oocytes were washed and then perfused with 3 μM GABA for different times prior to challenge. Increasing the pre-perfusion time from 1 to 12 min showed that the extent of current depression was dependent upon pre-perfusion time, but there were no significant differences between 9 and 12 min, suggesting that desensitization reaches a steady state within 9 min. This also illustrates that unlike rapid application techniques (18), gravity perfusion requires longer periods of equilibration to achieve a stabilized concentration of agonist in the pre-perfusion time. Following this application, the response to the challenge concentration was measured again. Increased pre-perfusion time significantly depressed GABA-gated current from control values, but there was no significant difference between the 9-min pre-perfusion time and the 12-min pre-perfusion time. Thus, in the oocyte recording system, long pre-perfusion times (9–12 min) were required to achieve a steady state desensitized response to the agonist. Data represent the mean ± S.E. of (n) independent experiments.

and, furthermore, that the extent of desensitization is dependent on the number of activations (see also Ref. 17). Comparison of the data in Fig. 1, A and B, demonstrates that the magnitude of steady state desensitization is also dependent upon the concentration of GABA in the perfusion medium (see below and Fig. 4).

Optimization of the Experimental Conditions to Investigate Desensitization

The experimental protocol used here to study GABA<sub>A</sub> receptor desensitization was adapted from that of Bartrup and Newberry (17) who used whole cell patch clamp techniques to study agonist-induced desensitization of the serotonin type 3 receptor in NG108-15 cells. Because GABA<sub>A</sub> receptor desensitization has not been quantified previously using the Xenopus oocyte system, we have optimized the experimental procedures to ensure that any desensitization observed was due to the presence of agonist in the perfusate and was not affected by other experimental variables.

Frequency of Challenge Application—The data in Fig. 2A illustrate the time interval between challenges required to avoid frequency-dependent effects in the wild-type receptor. As in Fig. 1, currents to a maximally effective GABA concentration (1 mM) were recorded at regular time intervals, but in these experiments, this interval was varied between 3 and 12 min as indicated. After the third control challenge, the oocyte was perfused with 3 μM GABA, a concentration that approximates its IC<sub>50</sub> for desensitization (see below). Responses to subsequent regular challenges continued to be recorded prior to washout of GABA from the perfusate. In these experiments, perfusion with the lower concentration of GABA was started during the recovery phase from the challenge application, i.e. immediately after receptor activation. Under these conditions, there was an immediate reduction in the current amplitude to the next challenge (cf. Fig. 1B), again demonstrating the activation dependence of this phenomenon (see below and also Ref. 17). As noted under “Experimental Procedures,” both preperfusion protocols gave the same steady state levels of desensitization.

By using a 9- or 12-min interval between challenges, the control responses were reproducible in amplitude, and there was no difference in the magnitude of the inhibition observed during agonist perfusion or in the recovery upon washout (Fig. 2A). A 3-min interval is clearly not suitable for measuring the concentration dependence of desensitization, because the receptor is unable to recover from the desensitization induced by the challenge itself. This is shown by the decrease in the amplitudes of successive control currents prior to inclusion of the lower concentration of GABA in the bath. By using a 6-min interval, there were apparent frequency-dependent effects on washout, i.e. the current did not immediately return to control levels. Similar control experiments have been carried out using muscimol (data not shown). In all cases, a 12-min interval between challenges gave rise to reproducible effects in which the apparent desensitization depends only on the presence of agonist in the perfusion medium and on the number (see e.g. Fig. 1B) but not on the frequency of channel activations.

Desensitization of GABA<sub>A</sub> Receptors Carrying Mutations of Tyr-62 of the β2 Subunit

Both mutant receptors desensitized upon bath perfusion with GABA as shown in Fig. 3. The data shown in this figure were obtained using perfusate GABA concentrations that approximated the IC<sub>50</sub> value for desensitization for each receptor (see below). In receptors carrying the β2 subunit Y62F mutation, the desensitization characteristics measured in control experiments (Fig. 3A) were very similar to those of wild-type receptors. However, notable differences were observed using the Y62S mutant (Fig. 3B). For this receptor, few frequency-

![Graph](https://via.placeholder.com/150)
dependent effects were apparent using challenge intervals varying from 3 to 12 min. This indicates that this receptor recovers more quickly (within 3 min) from the desensitization induced by the challenge than does the wild-type receptor.

**Effect of Agonist Concentration on Receptor Desensitization**

Experiments similar to those illustrated in Fig. 1 were carried out to investigate the effects of agonist concentration on the steady state level of desensitization. Fig. 4A shows the concentration dependence of both GABA- and muscimol-induced desensitization of the wild-type receptor, and curve fitting gave apparent IC$_{50}$ values of 3.3 and 0.7 M, respectively (Table I). In direct binding studies using [3H]muscimol, we have reported that the wild-type receptor expressed in tsA201 cells has (at least) two classes of binding sites with affinities of about 8 nM and 0.43 M (10). Although the latter value is subject to error due to rapid ligand dissociation from lower affinity sites during binding assays (see Ref. 13), the apparent K$_D$ value for this population correlates well with the IC$_{50}$ value for muscimol-induced desensitization. Our preliminary interpretation is that desensitization may be induced by occupancy of the lower affinity binding sites measured in radioligand binding studies.

The Y62F (Fig. 4B) and Y62S (Fig. 4C) mutations altered the concentration dependence for both GABA and muscimol-induced desensitization, the effects being greater in the case of the Y62S substitution (Table I). In both cases, muscimol was more potent than GABA in inducing desensitization. We have reported previously (10) that muscimol is also more potent in activation of these mutant receptors.

**Receptors Carrying the β2 Subunit Y62S Mutation Do Not Maintain the Desensitized State**

During the course of the above experiments, we observed several curious properties relating to the time dependence of desensitization of the Y62S mutant receptor. When the receptor was perfused with low concentrations of GABA (≤10 μM), i.e. below its IC$_{50}$ for steady state desensitization, the receptor appeared to desensitize and then recover from this desensitization.
zation even in the continued presence of the perfused agonist. The representative traces in Fig. 5 illustrate this phenomenon. In these experiments, the wild-type (Fig. 5A) or Y62S (Fig. 5B) receptors were challenged successively with concentrations of GABA equivalent to their EC_{50} values for activation (see Table I). In control experiments, we have shown that the steady state level of desensitization is independent of whether the EC_{50} (used in Fig. 5) or E_{\text{max}} (as in Figs. 1–4) concentration of agonist is used as the challenge application (data not shown). When the wild-type receptor was perfused with 3 μM GABA, the currents, as expected from the previous results, declined to a steady state level and returned to control values upon washout. In contrast, the Y62S mutant receptor showed desensitization during the pre-perfusion, but rather than reaching a steady state residual current, the currents began to grow again even in the continued presence of 3 μM GABA in the perfusate. Thus this receptor, which we have shown previously to lack measurable high affinity binding sites (10), appears unable to maintain the desensitized state. Fig. 6 quantifies this recovery phenomenon when the GABA concentration in the perfusate was increased to 10 μM. In the wild type (Fig. 6A), the receptor desensitized, and this state was maintained until washout of the GABA from the perfusate. In contrast, the Y62S mutant desensitized but then recovered to control values in the continued presence of perfused GABA (Fig. 6B). The recovery phenomenon in the mutant is concentration-dependent. Although it is clear at low perfusate concentrations, i.e. 3–10 μM (Figs. 5 and 6), it is less obvious at higher concentrations. This is discussed further below.

Rate of Recovery of Peak Amplitude as Function of Time

To define further the recovery of GABA_{A}Rs from desensitization, we have investigated the rate and extent of recovery using wild-type and mutant Y62S receptors. In these experiments, a desensitizing concentration of GABA was applied, and the amplitude of the peak current to a subsequent challenge administered at times varying from 1 to 30 min after the initial application was measured. Fig. 7 shows the complete recovery from desensitization of wild-type (Fig 7A) and mutant (Fig 7B) receptors from successive challenges at their respective EC_{50} concentrations. The Y62S mutant receptor recovers ~2-fold faster than the wild type, and curve fitting by a single exponential model (Table II) gave half-times for recovery of 0.8 and 1.5 min, respectively. These results are in agreement with the data presented in Figs. 2 and 3, which show that the mutant receptor recovers more quickly from desensitization induced by the challenge application.

TABLE I

| β2 subunit | IC_{50} (μM ± S.E.) | n_{H} | Mutant, wild-type |
|------------|---------------------|------|------------------|
| GABA       |                     |      |                  |
| Wild-type  | 3.3 ± 1.0           | −0.99 ± 0.16 | 1.0 |
| Y62F (4)   | 8.2 ± 1.1^a         | 1.05 ± 0.07 | 2.5 |
| Y62S (3)   | 24.7 ± 5.7^a        | 1.03 ± 0.05 | 7.5 |
| Muscimol   |                     |      |                  |
| Wild-type  | 0.7 ± 0.01          | −0.77 ± 0.08 | 1.0 |
| Y62F (3)   | 2.1 ± 0.3^a         | −0.86 ± 0.07 | 3.2 |
| Y62S (3)   | 3.8 ± 0.5^a         | −1.32 ± 0.34 | 5.7 |

^a p < 0.05.
^b p < 0.001.

Similar experiments were carried out in the presence of 3 μM bath-perfused GABA, i.e. similar to the conditions used in Fig. 5. In the case of wild-type receptors, 3 μM GABA did not significantly alter the rate of recovery, but at longer time intervals, the current induced by the challenge application was reduced, reflecting the ability of the bath perfused GABA to “lock” a proportion of the receptors in a desensitized state. As expected from the previous results, the Y62S mutant receptor displayed different recovery properties (Fig. 7B). The receptor initially desensitized, but despite the continued presence of bath-perfused GABA (3 μM), the magnitude of the currents returned to control levels (Fig 7B) within about 20 min. This again demonstrates that low concentrations of GABA in the perfusate are able to induce desensitization but are unable to maintain the receptor in a desensitized state. First impressions of the data in Fig. 7B suggest that the rate of recovery may be slowed in the presence of GABA in the perfusate. However, this is unlikely to be due to a direct effect on the kinetics of recovery from the desensitization induced by the challenge application itself because no such behavior was seen in other experiments, e.g. Fig. 3B. It is more likely that these data reflect the same phenomenon as depicted in Fig. 5B, i.e. that the slow recovery to control levels is dictated by the time scale of the reversal of the transient desensitization process.

DISCUSSION

The identification of molecular domains that contribute to receptor activation and desensitization is a primary goal in
structural studies of GABA\textsubscript{A}Rs. The number of agonist-binding sites on a single GABA\textsubscript{A}R has not been unequivocally defined, owing in large part to the inherent complexity of this receptor family. Early binding studies using native brain membranes suggested that there were at least two classes of binding sites of high ($K_D = 10 - 30$ nM) and low ($K_D = 0.1 - 1.0$ \mu M) affinity (11, 13). Although these observations may have arisen from the heterogeneity of GABA\textsubscript{A}Rs that are now known to exist in mammalian brain, more recent studies using recombinant, presumably homogenous, receptor subtypes suggest that individual receptors carry both high and low affinity sites for agonists (10, 20). In addition to these sites, which are amenable to measurement in direct binding studies, many investigators have suggested (11) that there may also be “ultralow” affinity sites ($K_D > 10$ \mu M) to explain the higher concentrations of GABA that are required to activate the GABA\textsubscript{A}R ion channel. Thus agonist binding is complex, and irrespective of the true number of binding sites carried by each receptor, it is clear that the functional roles of the multiple classes of sites have not been adequately addressed. In this report, we use site-directed mutagenesis to selectively disrupt the high affinity sites to probe their roles in receptor function.

The major goal of the present study was to relate the occupancy of binding sites to the effects on GABA\textsubscript{A}R desensitization. Although the \textit{Xenopus} oocyte perfusion system is not a rapid application system and, as such, does not allow biophysical analysis of the microscopic rates of desensitization, it appears well suited for this type of analysis in which the concentration dependence of desensitization is measured under essentially equilibrium conditions. The experimental procedures have been optimized (see Figs. 2 and 3) to avoid potential artifacts, and as discussed below, the system has been validated by the close agreement of a number of the present results with those reported for other receptor systems using more rapid perfusion and other techniques such as patch clamp analysis to study wild-type and mutant receptors with respect to desensitization (17, 21). Further analysis of these mutant receptors using rapid application techniques will be important in elucidating the detailed mechanisms underlying desensitization and recovery.

Several models of receptor activation and desensitization have been proposed to account for conformational transitions of allosteric proteins. Adaptations of the Monod-Wyman-Changeux model (22) suggest that within a population of receptors,
there exist two receptor conformations in which there is an equilibrium between a low affinity resting state and a high affinity desensitized state. This two-state model and its variants, which have been developed mainly to describe the transitions of the peripheral nAChR, propose that agonist binding to the resting state promotes both channel activation and desensitization and predict that the heterogeneity observed in binding studies arises from a dynamic equilibrium between different conformational states of the same receptor-ligand complex. Similarly, some investigators have suggested that the two populations of agonist sites detected in binding studies of GABARs also reflect the presence of interconvertible states. However, we and others (11) have suggested that the sites are independent, in accordance with our early model of distinct sites being involved in activation and desensitization of the Torpedo nAChR (see Ref. 23).

One of the basic predictions of two-state models, developed from the original Monod-Wyman-Changeux model, is that desensitization may occur without channel activation (for review see Ref. 29). In the present study, bath perfusion of agonist reproducibly inhibited GABA-gated chloride conductances at recombinant wild-type GABARs in a concentration-dependent manner. However, the present data suggest that desensitization may require channel activation. Agonist perfusion did not reduce the amplitude of the response to the first challenge application unless (a) the concentration of agonist in the perfusate was sufficiently high to cause significant receptor activation (Fig. 1A) and/or (b) agonist perfusion took place during the recovery phase from the desensitization induced by the agonist challenge, i.e. immediately after channel activation (Fig. 2A). Low concentrations of bath perfused agonist (e.g. 1 μM as in Fig. 1B) did not diminish the response to the first challenge even when the perfusion time was prolonged (data not shown). These observations are similar to those previously reported by Bartrup and Newberry (17) who used whole cell patch clamp techniques to study the concentration dependence of desensitization of the serotonin type 3 receptor in NG108-15 cells. Such activation dependence is also evident in the current results reported by Corringer et al. (24) investigating the desensitization properties of an α7 nAChR-serotonin type 3 chimera expressed in Xenopus oocytes. In one chimera, 10 nM nicotine did not reduce the amplitude of the response to the first 1 μM nicotine challenge (approximately the EC50 for this mutant receptor), but subsequent challenge responses were progressively reduced to a steady state level. A higher perfusate concentration (0.1 μM) caused an immediate inhibition, possibly because some receptor activation had occurred during the perfusion.

The concentration dependence for desensitization of wild-type receptors by muscimol and GABA gave IC50 values of 0.7 and 3.3 μM, respectively (Fig. 4A). The latter value agrees well with the estimate of 1–2 μM reported by Overstreet et al. (25) for GABA-induced desensitization of native receptors in hippocampal slices. Measured IC50 values are also in agreement with estimates of the affinities of the lower affinity population of sites measured in binding studies. We reported previously that the lower affinity (Kd = 1 μM muscimol binding to this receptor subtype expressed in tsA201 cells is 0.43 μM) (10). The affinity for GABA binding to this population of sites was ~1 μM, as measured indirectly from its ability to potentiate [3H]flunitrazepam binding. Whereas this correlation may be fortuitous, it is clear that the IC50 values do not correlate with the affinities of the high affinity sites measured under equilibrium conditions (Kd of 8.1 and 119 nM for muscimol and GABA, respectively). The association of desensitization phenomena with the lower affinity sites contradicts the speculation of many investigators that it is the high affinity agonist-binding sites that mediate desensitization (see Ref. 27).

Intriguingly, we recently obtained a very similar result when studying the nAChR from Torpedo electric organ but using a quite different technique, i.e. rapid agonist-induced flux measurements using native Torpedo membrane vesicles (28). Under equilibrium conditions, it is well established that this receptor carries two high affinity sites for agonists (Kd of ~100 nM for carbachol), and it is generally assumed that occupancy of these sites in the resting state of the receptor leads to the equilibrium desensitized state (29). However, saturation of these sites with carbachol under equilibrium conditions did not diminish the flux response induced by subsequent exposure to a higher (activating) concentration of this ligand. Thus, as for the GABAR receptor above, occupancy of the high affinity sites per se does not desensitize the receptor. In contrast, the concentration dependence for desensitization, measured by the inhibition of the flux response upon subsequent challenge, gave an EC50 for carbachol of 15.5 μM. This correlates well with occupancy of intermediate affinity sites that we have so far detected only under non-equilibrium conditions (30, 31). The parallels between the two receptor systems suggest that the underlying mechanisms for agonist-induced desensitization may be common to all members of this receptor family.

Desensitization of the two mutant receptors, Y62F and Y62S, occurred at 2.5–7.5-fold higher concentrations of both GABA and muscimol, with the Y62S mutant being more affected. These decreases in apparent affinity parallel those of these mutations on agonist-induced receptor activation (10). In control experiments, the desensitization properties of the Y62F-containing receptor were otherwise very similar to those of the wild-type. We reported previously (10) that this mutation has a mixed effect on [3H]muscimol binding, reducing the two populations of sites observed in the wild-type receptor to a single resolvable population of intermediate affinity. Thus, it would appear that the changes in agonist binding affinity may be due to perturbation of components of desensitization, which could account for the decrease in agonist sensitivity.

The data in Fig. 3B were the first indications that other desensitization characteristics of the Y62S mutant receptor were different from their wild-type counterparts because this receptor appeared to recover from desensitization more quickly, i.e. within about 3 min under the experimental conditions employed. However, the most interesting property of this mutant is illustrated in Figs. 5B and 6B, i.e. that low concentrations of agonist desensitize the receptor, but this reverses despite the continued presence of agonist in the perfusate. The initial desensitization observed is clearly due to the presence of GABA in the perfusate. In Fig. 5B, it is shown that under

| Table II |
| --- |
| Rates of recovery for recombinant GABA R receptors carrying either the wild-type or mutant β2 subunits when expressed in Xenopus oocytes |

Data represent the mean ± S.E. for (n) independent experiments performed as described. Mean k and Y∞ values were compared using an unpaired Student’s t test to compare values at the EC50 and values at the EC50 in the presence of GABA.

| (GABA challenge) | k min⁻¹ ± S.E. | Y∞ % ± S.E. |
| --- | --- | --- |
| α1β2γ2 | 47.3 ± 4.7 | 96.2 ± 2.5 |
| α1β2γ2 (3) | 63.6 ± 8.3 | 64.2 ± 1.7* |
| α1β2Y62Sγ2 | 84.1 ± 9.2 | 98.8 ± 1.9 |
| α1β2γ2 + 3 μM GABA perfusion (3) | 20.3 ± 2.4* | 92.9 ± 3.4 |
| α1β2γ2 | 4.7 ± 0.4 | 96.2 ± 7.1 |
| α1β2γ2 + 3 μM GABA perfusion (3) | 2.5 ± 0.3 | 92.9 ± 3.4 |

* p < 0.001.
+ p < 0.01.
control conditions, the currents induced by the challenge concentration fully recover within the first 9-min assay point used. Because the main difference between the wild-type receptor and the β2 Y62S mutant is the presence of high affinity sites in the former and their absence in the latter, we conclude that it is the agonist occupancy of the high affinity sites that stabilizes the equilibrium desensitized state.

The above results have provided new insights into possibly conserved mechanisms of this receptor family. However, many quantitative issues remain to be resolved. The number of agonist sites on a single receptor molecule has not been established, and the likely allosteric interactions between sites of different affinities inevitably complicate analysis and interpretation. In binding studies, the Y62S mutant receptor appeared to carry only one population of sites for GABA with an estimated $K_D$ of about 1 μM (10), i.e. not significantly different from the lower affinity sites measured in the wild-type receptor. Similar GABA concentrations were required to induce transient desensitization of the Y62S mutant (see Figs. 5 and 6) suggesting that, as in the wild type, it is the occupancy of these sites that initiates the desensitization process. However, in the case of the mutant receptor, in which the high affinity sites are disrupted, the desensitized state is not “locked,” and the receptor recovers. As noted under “Results,” this recovery phenomenon is observed only at relatively low perfusate concentrations (1–10 μM). At higher concentrations, the observed desensitization displays similar properties to that of the wild type, albeit with a higher IC$_{50}$ value (24.7 μM for GABA; Table I). This value does not correspond to either the apparent affinity measured in equilibrium binding studies or the EC$_{50}$ for activation (178 μM) (10). Indeed for all three receptors studied here, desensitization occurred at concentrations that were ~10-fold lower than those required for receptor activation. It remains to be established whether this reflects the presence of distinct low affinity sites that mediate channel opening as we have proposed for the Torpedo nAChR (see Ref. 28).

In conclusion, we have investigated the functional consequences of mutations to the high affinity agonist-binding sites of recombinant GABA$_A$Rs expressed in Xenopus oocytes. Desensitization of the wild-type α1β2/2 receptor has been characterized with respect to agonist concentration dependence, rate of recovery, and apparent dependence on receptor activation. The reduction in affinity for [3H]muscimol that occurs upon substituting Tyr-62 of the β2 subunit by phenylalanine and serine (10) is paralleled by an increase in the concentration dependence of agonist-induced desensitization. The most intriguing observation is that the Y62S mutant receptor, which lacks measurable high affinity binding sites, recovers from desensitization even in the continued presence of agonist. This suggests that the high affinity binding site plays a major role in conferring stability to the desensitized state, perhaps by virtue of a “locking” mechanism. Confirmation of this novel result will undoubtedly require crystallization of native GABA$_A$R and the use of high resolution biophysical techniques to monitor conformational transitions. Nevertheless, in the present study, we have begun to unravel the importance of high affinity agonist binding domains in GABA$_A$R function.

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J. Glen Newell and Susan M. J. Dunn

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