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Agonist-induced closure of constitutively open γ-aminobutyric acid channels with mutated M2 domains

($GABA_C$ receptors/site-directed mutagenesis/*Xenopus* oocytes)

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ABSTRACT **Ligand-gated ion channels display a fundamental property—channels remain virtually closed at rest and open upon agonist binding. Here we show that substituting alanines for either of two amino acid residues (T314 or L317) in the M2 region of the γ-aminobutyric acid (GABA) ρ1 subunit results in spontaneous channel opening in the absence of ligand. Surprisingly, for two single point mutants (T314A or L317A), application of very low concentrations of agonist partially suppressed this spontaneous current, while higher concentrations re-activated the receptors. When both of these sites were mutated (T314A/L317A), GABA nearly completely suppressed the constitutive current and did not re-activate the current even at very high concentrations. This study provides important new insights into the structure–function relationship of ligand-gated ion channels, where modification of the structure of the channel pore region not only alters the allosteric transition of the receptor protein but also reverses the polarity of agonist regulation of channel gating. Our results suggest that the sites where these two residues are located are structurally critical for channel gating.**

Members of the ligand-gated ion channel superfamily share certain structural and functional similarities (1–5) and include the following receptors: nicotinic acetylcholine (nACh), glycine, 5-hydroxytryptamine type 3, and γ-aminobutyric acid (GABA, A and C subtypes). In this family, the receptor is apparently composed of five subunits, and the second membrane-spanning segment (M2) forms the pore of the channel (6–8). Typically, the channel is closed at rest and opens upon agonist binding to its receptor site, located at some distance from the pore region. Channel activity is thought to be controlled by a gate inside the pore (7–9). The structure of the nACh receptor (nAChR) channel in the closed and open states has been characterized at 9-Å resolution in electron crystallographic studies (9, 10). Receptor protein function has been interpreted in terms of an allosteric transition model (11, 12). However, the nature of the channel gate as well as the exact mechanism of ligand regulation of gating are still not clear.

The GABA ρ subunits are known to comprise, at least in part, the recently described $GABA_C$ receptor (5, 13–17). A threonine residue lies at position 314 in the M2 domain of the ρ1 subunit and is conserved in most known GABA and glycine receptor subunits (Fig. 1). Previously, we reported that a naturally occurring mutation (a methionine instead of a threonine) at the corresponding site of the ρ2 subunit is responsible for the resistance to picrotoxinin (PTX) blockade of native $GABA_C$ receptors in the rat retina (16). To explore further the role of this and surrounding sites on receptor-channel function of $GABA_C$ receptors, we made a series of additional substitutions by site-directed mutagenesis, taking advantage of the observation that recombinant ρ1 subunits are in general capable of forming functional homomeric receptors (5, 16). Here we report the unexpected finding that mutations of this site, in addition to an adjacent site facing the pore, lead not only to channel opening in the absence of ligand but also to reversal of agonist action on channel gating—GABA, at low concentrations, now turns the channels off rather than on.

nACh α	241	E	K	M	T	L	S	I	S	V	L	L	S	L	T	V	F	L	L	V	I	V	E	262
GABA α1	254	A	R	T	V	F	G	V	T	T	V	L	T	M	T	T	L	S	I	S	A	R	N	275
Glycine α1	258	A	R	V	G	L	G	I	T	T	V	L	T	M	T	T	Q	S	S	G	S	R	A	279
GABA ρ1	307	A	R	V	P	L	G	I	T	T	V	L	T	M	S	T	I	I	T	G	V	N	A	328
									314			317													

FIG. 1. Alignment of residues in the second membrane-spanning (M2) region of the α subunit of the nAChR (*Torpedo*), the α1 subunit of the $GABA_A$ receptor, the α1 subunit of the glycine receptor, and the rat ρ1 subunit of the $GABA_C$ receptor. The boxed residues in the α subunit of the nAChR and the α1 subunit of the $GABA_A$ receptor have been proposed to face the lumen of the channel (18–20).

MATERIALS AND METHODS

Site-Directed Mutagenesis. As we have previously described (16), point mutations were introduced into the rat ρ1 cDNA clone by using the Chameleon double-stranded, site-directed mutagenesis kit based on *T7* DNA polymerase (Stratagene) and confirmed by sequencing of the mutated region. For the ρ1(T314A) mutant, the entire open reading frame was sequenced. Capped cRNA was transcribed *in vitro* by T3 polymerase using the Ambion (Austin, TX) mMessage mMachine kit.

Electrophysiological Recordings of Recombinant Channels from Oocytes. The electrophysiological methods were similar to those that we have described (16). In brief, stage V and VI *Xenopus laevis* oocytes were harvested and defolliculated. Twenty to 50 ng of cRNA dissolved in 50 nl of nuclease-free water were injected into oocytes. Injected oocytes were incubated at 18°C in ND96 medium (96 mM NaCl/2 mM KCl/1.8 mM $CaCl_2$/1 mM $MgCl_2$/10 mM Hepes, pH 7.5), and supplemented with 2 mM sodium pyruvate and 50 units/liter gentamicin.

Whole-cell recordings were performed 2–7 days after cRNA injection using a dual-electrode voltage-clamp amplifier (Warner Instruments, Hamden, CT; model OC-725A). Micropipettes were filled with 3 M KCl. Oocytes were voltage-clamped at −70 mV during recordings unless otherwise indi-

Abbreviations: nACh, nicotinic acetylcholine; nAChR, nACh receptor; GABA, γ-aminobutyric acid; WT, wild type; PTX, picrotoxinin; 3-APMPA, 3-aminopropy[methyl]phosphinic acid.

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cated. The frog Ringer's solution contained 115 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, and 10 mM Hepes, adjusted to pH 7.2. Drugs were applied to oocytes via a rapid superfusion system. In our system, a full exchange of external bath solution was achieved in less than 30 s.

$[Na^+]_o$ and $[K^+]_o$ substitutions were made by *N*-methyl-D-glucamine and Na^+, respectively. Substitution of $[Ca^{2+}]_o$ was made by Mg^{2+} (in the case of decreases) or Na^+ (in the case of increases). To determine reversal potential, voltage-ramps from a holding potential of −70 mV to a target potential of +30 mV were performed over a 1.5-s interval after (>100 s) complete exchange of the extracellular solution. Reversal potentials were determined after subtraction of leakage currents. For the wild-type (WT) receptor, leakage currents were obtained by identical voltage-ramp protocols performed prior to receptor activation. For ρ1(T314A) receptors, leakage currents were constructed from the mean leakage current of WT receptors expressed in the same batch of oocytes ($n > 5$) with similar extracellular ionic concentrations. For ρ1(T314A) receptors, reversal potentials were also determined by subtracting currents obtained in either the presence or absence of 200 μM PTX. The reversal potentials obtained with these two methods for the mutant receptors were not significantly different and were therefore averaged together.

Single-channel recordings were performed in the outside–out patch configuration using an EPC-7 amplifier (List Electronic, Darmstadt, Germany). Before recordings, oocytes were manually devitelinized. The bath solution contained 140 mM NaCl, 0.5 mM $CaCl_2$, 1.5 mM $MgCl_2$, and 10 mM Hepes, adjusted to pH 7.4. Electrodes were coated with Sylgard and had resistances of 5–10 MΩ when filled with the following solution: 140 mM KCl, 2 mM $MgCl_2$, and 10 mM EGTA, adjusted to pH 7.4. Single-channel data were stored in a PCM recorder and analyzed with the PCLAMP 6 software program (Axon Instruments, Foster City, CA).

RESULTS

Constitutive Current in GABA ρ Receptors Mutated in the M2 Domain. When we mutated the threonine at position 314 to an alanine, oocytes injected with ρ1(T314A) cRNA displayed a spontaneous inward current when voltage-clamped at −70 mV (Fig. 2*A*). The magnitude of the current ranged from 0.3 μA to 1.2 μA (0.61 ± 0.22 μA; mean ± SD, $n = 114$). Oocytes injected with WT ρ1 cRNA, water, or nothing at all did not display this current, indicating that the spontaneous current was due to the expression of the mutated subunits. The spontaneous current was not blocked by 100–500 μM 3-APMPA, a competitive $GABA_C$ receptor antagonist (21, 22). In contrast, PTX, a blocker of ligand-gated Cl^- channels, suppressed the current in a dose-dependent manner, as indicated by a reduction in spontaneous current (Fig. 2*A*). The IC_{50} value of the PTX dose-inhibition curve was 14.2 μM (Fig. 2*B*). For the WT ρ1 receptor, we previously reported that the PTX dose-inhibition curve was dependent on GABA concentration, and the IC_{50} values of PTX on 1, 2, and 20 μM GABA-evoked responses were 0.52, 1.1, and 40.0 μM, respectively (16). Taken together, our data indicate that a single-point mutation in the channel domain of the ρ1 receptor resulted in channel opening in the absence of ligand.

We found that the voltage-current relationship for the PTX-suppressible spontaneous current was linear (Fig. 2*C*). The reversal potential varied between oocytes of different batches. However, comparing oocytes from the same batch, the reversal potential for the spontaneous current of the ρ1(T314A) receptor (−39.0 ± 0.8 mV, mean ± SD; $n = 11$) in normal frog Ringer's solution was somewhat more negative than that of the GABA-evoked current of the WT ρ1 receptor (−33.2 ± 1.8 mV, mean ± SD; $n = 5$). Since mutations in the M2 domain of nACh receptors have been reported to alter

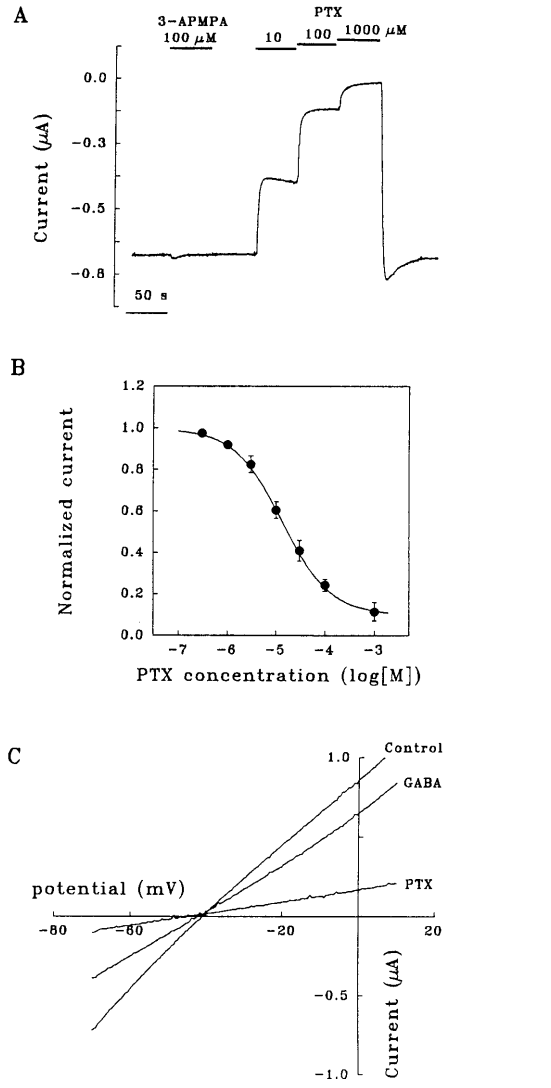

FIG. 2. Channel block and ionic permeability of the spontaneous current of homomeric ρ1(T314A) receptors expressed in *Xenopus* oocytes. (*A*) Typical current displayed in an oocyte injected with ρ1(T314A) cRNA. Application of 100 μM 3-aminopropy[methyl]phosphinic acid (3-APMPA) had no effect on this current. PTX (10–1,000 μM) blocked the spontaneous inward current in a dose-dependent manner, as indicated by the appearance of an apparently outward current. (*B*) Normalized PTX dose-inhibition curve for ρ1(T314A) receptors. Data points represent mean ± SD ($n \geq 4$ oocytes). The line is the fit to the Hill equation, $I = I_{max}/(1 + \{[A]/IC_{50}\}^{n_H})$, where [A] is the PTX concentration and n_H is the Hill coefficient. The fit yielded an IC_{50} and Hill coefficient of 14.2 μM and 0.9, respectively. (*C*) Typical current–voltage relationship of the spontaneous current recorded from an oocyte injected with ρ1(T314A) cRNA in the absence of ligand, in the presence of 0.1 μM GABA, and in the presence of 200 μM PTX in normal frog Ringer's solution.

ionic selectivity (23, 24), we determined whether or not the small difference in the reversal potentials was due to a change in ionic selectivity of the mutant receptor channel. However, we found that reducing $[Na^+]_o$ from 115 to 10 mM or increasing $[Ca^{2+}]_o$ from 1.8 to 10 mM did not change the reversal potential significantly [respective changes were 1.8 ± 2.0 mV and 0.1 ± 0.7 mV (mean ± SD; $n = 5$ for each)]. Varying $[K^+]_o$ from 0 to 10 mM also did not change the reversal potential significantly (1.3 ± 1.4 mV; $n = 5$). These results suggest that a substantial permeability of the mutant channel to these cations is very unlikely. Thus, the mutant receptor channel appears to remain predominantly anion selective. The more negative reversal potential of the ρ1(T314A) receptor com-

pared with WT is probably due to a lower intracellular Cl^- concentration in oocytes expressing the mutant receptor because of the steady efflux of anions through its spontaneously open channels.

Agonist-Induced Channel Closure. Quite unexpectedly, application of GABA at extremely low concentrations suppressed the spontaneous current of the mutant ρ1(T314A) receptor. The inhibitory effect was apparent even at low nanomolar concentrations of GABA (Fig. 3*A*, initial two traces). Additionally, at a concentration of 1 μM or more, two GABA-evoked components could be clearly resolved, an initial suppression of the spontaneous current followed by an inward current (indicated by the first arrow in Fig. 3*A*). However, at high GABA concentrations (for example, 100 μM), activation of the inward current became so fast that the first inhibitory component became undetectable (indicated by the second arrow in Fig. 3*A*). This inward current was transient in nature. The decline of the inward current also became more rapid at higher GABA concentrations, as evidenced by comparing the currents indicated by the two arrows in Fig. 3*A*. The steady-state GABA dose-response curve, measured when the effect of GABA had reached a plateau, is illustrated in Fig. 3*B*. The fact that GABA never completely suppressed the spontaneous current and that the GABA dose-response curve flattened out at [GABA] $>$ 0.1 μM is likely due to the appearance of the inward current. Fitting the dose-inhibition data at low concentrations of GABA ($\leq$0.1 μM) to the Hill equation yielded an IC_{50} value of 0.11 μM and a Hill coefficient of 0.8. The WT ρ1 receptor does not manifest a detectable response to GABA at concentrations $\leq$ 0.1 μM and its GABA dose-response curve has an EC_{50} of 1.1 μM and a Hill coefficient of 2.3 (Fig. 3*C*) (16). Finally, both components induced by low and high GABA concentrations were abrogated by 3-APMPA (Fig. 3*D*). This result indicates that the suppression of the spontaneous current was mediated by GABA binding at the receptor's agonist site.

Over a wide range of concentrations, GABA-induced suppression of the spontaneous current varied linearly with voltage in the mutant ρ1(T314A) receptor (Fig. 2*C*). Removing extracellular Ca^{2+} affected neither the spontaneous nor the GABA-evoked currents, indicating that a Ca^{2+}-activated Cl^- current was not involved (data not shown). Furthermore, application of GABA (at concentrations ranging from 0.1 to 100 μM) did not cause a measurable shift in the reversal potential of the spontaneous current (Fig. 2*C*).

At either low or high concentrations, GABA-induced suppression of the spontaneous current was reversible, but the recovery period was prolonged. Desensitization states of ligand-gated receptors have been reported to display higher affinity for agonist-bound than resting (closed) or active (open) states (25). To determine whether or not the suppression of spontaneous current by GABA was due to receptor desensitization, we compared the effect of pre-application of GABA at concentrations of 0.1 or 1 μM on subsequent responses to high (100 μM) GABA. Although prolonged application of 0.1 μM GABA (150 s) produced an apparent maximal inhibition of the spontaneous current of the ρ1(T314A) receptor, such inhibition did not significantly re-

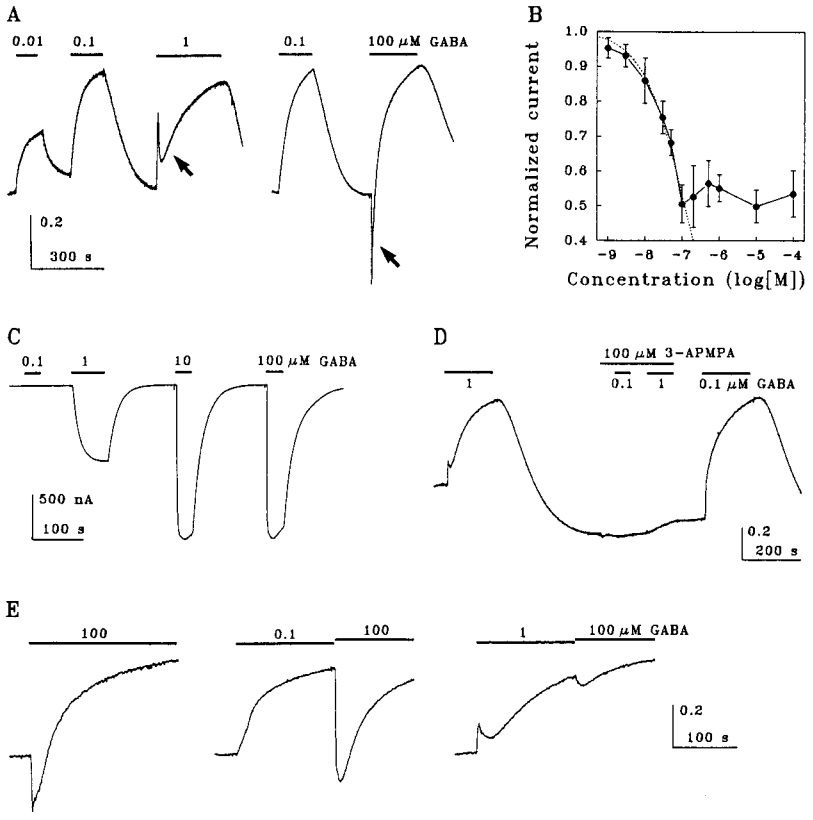

FIG. 3. Responses of M2 domain mutant ρ1(T314A) and WT receptors to GABA. (*A*) Typical responses of mutant ρ1(T314A) homomeric receptors to GABA. Two traces are illustrated from two different oocytes. GABA concentrations $>$0.1 μM produced an additional transient inward current, indicated by arrows. (*B*) Normalized GABA dose-response relationship for ρ1(T314A) receptors. Measurements were made after the effect of GABA had reached a plateau (steady state). Data points represent mean $\pm$ SD ($n \geq 5$ oocytes). For [GABA] $\leq$ 0.1 μM, the dotted line is the fit to the Hill equation. The fit yielded an IC_{50} and Hill coefficient of 0.11 μM and 0.8, respectively. (*C*) Typical responses of WT ρ1 receptors to GABA. GABA ($\leq$0.1 μM) did not evoke any detectable current. (*D*) Inhibitory effects of GABA (0.1 and 1 μM) on the spontaneous current of the ρ1(T314A) receptor were blocked by 100 μM 3-APMPA. (*E*) Demonstration that suppression of current produced by 0.1 μM GABA is not due to receptor desensitization. Three GABA responses on ρ1(T314A) receptors were recorded from different oocytes. Traces in *A*, *D*, and *E* are normalized to the amplitude of the initial spontaneous current, and thus the ordinate values represent arbitrary units.

duce the magnitude of the subsequent inward current evoked by 100 μM GABA (Fig. 3*E Left* and *Center*). In contrast, application of 1 μM GABA, which elicited an inward current, significantly reduced the inward current evoked by 100 μM GABA (Fig. 3*E Right*). Desensitization appears to underlie the apparent inward current induced by high (>0.1 μM) levels of GABA for two reasons: (*i*) application of higher concentrations of GABA (100 vs. 1 μM) favored more rapid fading of the inward current—i.e., the kinetics of fading were concentration dependent (see Fig. 3*A*), and (*ii*) subsequent to the addition of 1 μM GABA (but not of 0.1 μM), application of high concentrations of GABA (100 μM) induced a significantly smaller current (Fig. 3*E Right* trace versus the *Center* trace). In contrast, the decrease in spontaneous current seen with lower concentrations of GABA (0.1 μM) does not appear to be due to desensitization because a large inward response was elicited by subsequent application of a high (100 μM) concentration of GABA (Fig. 3*E Center* trace). Therefore, the response to low concentrations of GABA appears to be due to agonist-induced closure of the spontaneous inward current manifest by the mutant (Fig. 3*E Center* trace).

Nature and Position of the Amino Acid Residues Producing Constitutive Currents. Next, we studied the effect on channel closure during the resting state of the side chains of amino acid substitutions at position 314 of the ρ1 subunit. The threonine at position 314 was replaced with a valine or leucine, residues that are as hydrophobic as alanine but have larger side-chain volumes. An additional mutant was made by substituting a glycine for the threonine, producing a neutral residue but with a smaller side-chain volume. All of these mutants expressed functional receptors responding to GABA but none of them displayed the spontaneous current observed in ρ1(T314A) receptors (data not shown). Therefore, neither the hydrophobic nature nor the small side-chain volume of alanine alone is responsible for the effect, but together, both properties may be important.

To determine whether mutations at other positions in addition to T314 in the M2 domain could also result in constitutive channel opening, we constructed a series of such mutations. The two adjacent amino acid residues, isoleucine (I313) and threonine (T315), were replaced by alanines. In addition to T314, five other residues have been proposed to lie in or near the lumen of the channel (P310, L317, T318, T321, and T324 in Fig. 1) (18, 19). Hence, we also individually substituted each of these residues for alanines. Of these mutants, only ρ1(L317A), replacing leucine by alanine at position 317, also displayed spontaneous current in the absence of ligand.

The magnitude of the spontaneous current of the ρ1(L317A) receptor was comparable to that of the ρ1(T314A) receptor. Similar to ρ1(T314A), GABA responses of the ρ1(L317A) receptor manifest two components. Low concentrations of GABA partially suppressed the spontaneous current, while higher concentrations elicited an inward current (Figs. 4*A*). The GABA dose-response curve, measured at steady-state, is shown in Fig. 4*B*. However, for the ρ1(L317A) receptor, the inward current evoked by high concentrations of GABA did not fade, suggesting a lack of desensitization in this mutant (Fig. 4*A*). This finding is consistent with the interpretation of the data obtained with the ρ1(T314A) receptor that low GABA concentration-induced suppression is due to channel closure rather than receptor desensitization.

Additionally, we mutated both residues 314 and 317 to alanines, resulting in the double mutant ρ1(T314A/L317A). Oocytes injected with ρ1(T314A/L317A) displayed even larger spontaneous currents. Moreover, in this mutant, unlike the single amino acid residue mutations [ρ1(T314A) or ρ1(L317A)], GABA was found to close the receptor channels almost completely and not re-open them, even at high (millimolar) concentrations (Figs. 4*C*). Fitting the dose-inhibition data to the Hill equation yielded an IC_{50} value of 0.17 μM and a Hill coefficient of 2.7 (Fig. 4*D*).

Single-Channel Currents of WT and Mutant GABA ρ1 Receptors. We recorded single-channel currents from two of the mutant receptors, ρ1(T314A) and ρ1(T314A/L317A), and ρ1 WT receptors in the outside–out patch configuration. For

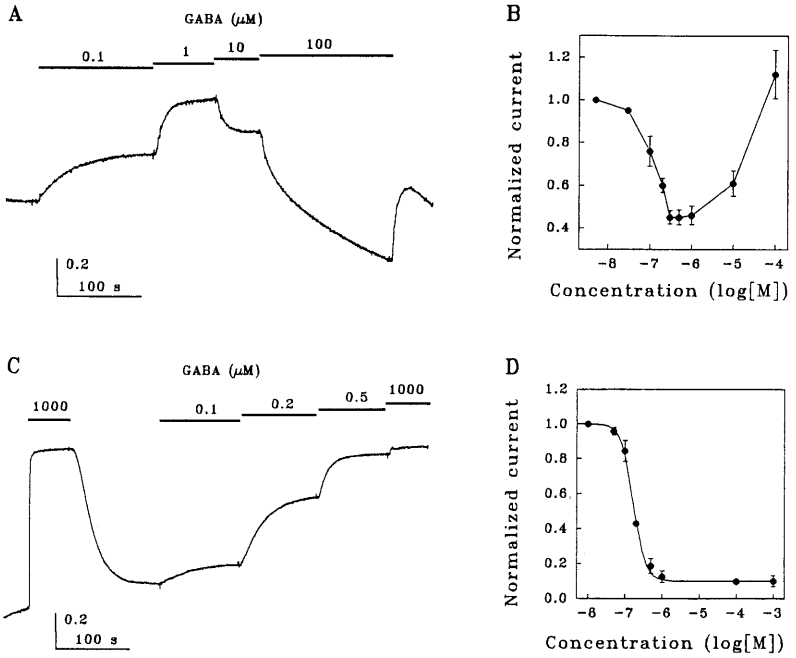

FIG. 4. Responses of M2 domain mutant receptors ρ1(L317A) and ρ1(T314A/L317A) to GABA. (*A*) Typical responses of mutant ρ1(L317A) receptors to GABA. (*B*) GABA dose-response relationship for ρ1(L317A) receptors. Measurements were made when the effect of GABA had reached a plateau (steady-state). Data points represent mean ± SD ($n \geq 5$ oocytes in each case). (*C*) Typical responses of mutant ρ1(T314A/L317A) receptors to GABA. (*D*) GABA dose-inhibition relationship for ρ1(T314A/L317A) receptors. Data points represent mean ± SD ($n \geq 5$ oocytes). The line is the fit to the Hill equation with $IC_{50} = 0.17$ μM and $n_H = 2.7$. Traces in *A* and *C* are normalized to the amplitude of the initial spontaneous current, and the ordinate values represent arbitrary units.

the WT ρ1 receptor, no spontaneous single-channel activity was observed in the absence of ligand (data not shown). Application of GABA (2 μM) evoked single-channel activity with multiple conductances ranging from 1 to 5 pS (n = 6 patches) (Fig. 5*A*). Channel openings lasted hundreds of milliseconds, consistent with the report of single-channel currents of $GABA_C$ receptors in rat bipolar cells (14). As expected, for both the ρ1(T314A) and ρ1(T314A/L317A) mutants, substantial channel activity was observed in the absence of ligand (Fig. 5*B* and top four traces of Fig. 5*C*; n = 5 patches for each mutant). In particular, for the ρ1(T314A/L317A) mutant, channels were predominantly open and only occasionally closed in the absence of ligand (Fig. 5*C*, top four traces). In contrast, after 2 μM GABA channels were mostly closed and only occasionally opened (Fig. 5*C*, bottom four traces).

DISCUSSION

We report here that mutations in the M2 region of the GABA ρ1 receptor resulted in activation of a substantial Cl^--selective conductance in the absence of agonist (i.e., a spontaneous or constitutive current). More interestingly, for two single point mutants (T314A or L317A), application of very low concentrations of agonist suppressed this spontaneous current, while higher concentrations re-activated the receptors. However, when both of these sites were mutated, GABA bound to the receptor to suppress the constitutive current but did not re-activate the current even at very high concentrations. We also show in single-channel recordings that the mutant GABA ρ1 receptors display a high probability of spontaneous channel openings, consistent with the large macroscopic constitutive current observed in whole-cell recordings. Our findings on GABA ρ1 receptors provide new insights into the understanding of the structure–function relationship of ligand-gated ion channels and the mechanism of ligand regulation of channel gating.

How might one interpret channel openings in the absence of ligand, and closure of channels in the presence of agonist, as

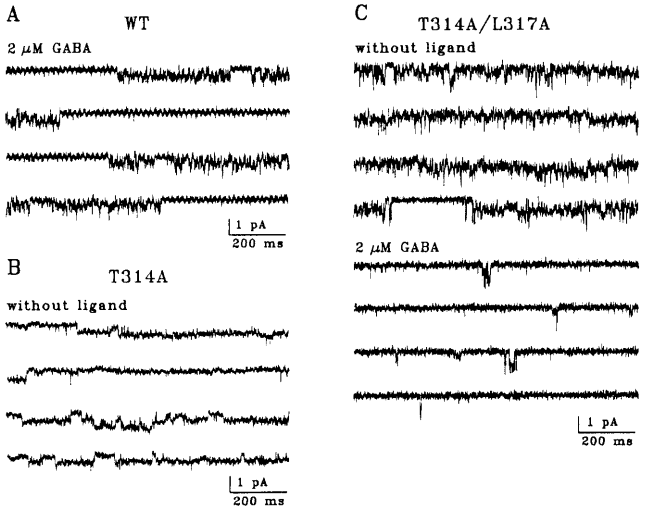

FIG. 5. Representative recordings of single-channel currents from WT ρ1, mutated ρ1(T314A), and mutated ρ1(T314A/L317A) receptors. Recordings were performed on outside–out patches pulled from oocytes at a holding potential of −100 mV. (*A*) GABA evoked single-channel currents from WT ρ1 receptors. (*B*) Single-channel currents of mutated ρ1(T314A) receptors in the absence of ligand. (*C*) Single-channel currents of mutated ρ1(T314A/L317A) receptors in the absence of ligand (top four traces) or in the presence of 2 μM GABA (bottom four traces). Data were sampled at 10 kHz and filtered at 0.5 kHz. The first two and last two traces in *A*, and all four traces in *B* and *C* (*Upper* and *Lower*) represent continuous recordings.

observed in these mutated GABA ρ1 receptors? Previously, occasional spontaneous channel openings had been observed in other receptors, including native nAChRs (26), and mutations in the M2 domain of nAChRs had been reported to increase the frequency of spontaneous channel openings (27, 28). Ligand regulation of channel gating has been explained in terms of classic allosteric transition theory (11, 12). One of the key features of the allosteric model is the interpretation of unliganded channel openings (29–31). The unliganded or spontaneous channel openings that we observed in mutated GABA ρ1 receptors and the agonist-induced closure of these channels are consistent with the allosteric interaction of ligand and receptor–protein. That is, agonists can produce negative allosteric effects as well as positive allosteric effects.

Also of interest, our results reveal that the mutations altered a number of other properties of GABA ρ1 receptors. At least for ρ1(T314A) receptors, there is marked receptor desensitization that is not present in WT receptors (13, 16). Previously, mutations have been reported in the M2 region that alter receptor desensitization in nACh and 5-hydroxytryptamine type 3 receptors (32, 33). One of the explanations for the effect on receptor desensitization is a change in ion channel activity (32). Furthermore, for all three of the mutants that we studied here, the apparent affinities for GABA are significantly increased compared with WT ρ1 receptors. Such an increase in the apparent affinity could be due to an increase in agonist binding affinity or to an alteration in channel gating. Ligand binding site(s) are known to be at some distance from the channel pore region (9). Hence, it would be somewhat surprising if a mutation in the channel pore region resulted in an alteration in the ligand binding affinity, although this possibility cannot be totally excluded. Alternatively, mutations in the channel pore region have been reported to increase apparent agonist affinity without altering ligand-binding affinity because of a change in the properties of channel gating (e.g., producing longer open times in response to the same concentration of agonist) (34).

Taken together, the phenotypes of the point mutations that we observed may have occurred as a result of a change in ion channel activity, as predicted by the allosteric model (31). Specifically, for all three mutants studied here, ρ1(T314A), ρ1(L317A), and ρ1(T314A/L317A), the mutations may convert one of the unliganded-closed states to an open state. In addition, for the double mutant, ρ1(T314A/L317A), the liganded-open state may become a nonconducting closed state(s). This interpretation has the advantage of conceptualizing both unliganded-open channels and reversal of agonist action (i.e., ligand-induced closure of channels).

Furthermore, the concentration-dependent actions of GABA on the ρ1(T314A) and ρ1(L317A) mutant receptors are likely due to discrete events, each involving the binding of an agonist molecule to the receptor. During agonist activation, receptor–channel complexes are thought to progress through multiple conformational states, presumably reflecting receptor binding of more than one agonist molecule with different intrinsic affinities (25). The presence of multiple high- and low-affinity ligand binding sites has been well characterized in receptor binding studies (35, 36) as well as in channel gating studies (37, 38). Specifically for the mutant ρ1(T314A) receptor, the nanomolar concentrations of GABA that produce suppression of the spontaneous current are comparable to high-affinity K_d values, while the Hill coefficient for the GABA dose-inhibition curve is less than one. Therefore, these results suggest that the suppression of the spontaneous current by low concentrations of GABA on the T314A mutant receptor may be due to the binding of a single agonist molecule. Such single-liganded receptor proteins may stabilize the closed conformational state of the mutant channel. In contrast, the current evoked by high concentrations of GABA on both the ρ1(T314A) and ρ1(L317A) mutants may be due to the binding

of two (or more) GABA molecules at the receptor, resulting in the further transition of the receptor to the liganded-open conformational state(s).

What influence on structure might these point mutations have to produce such an effect? It is commonly believed that channel activity is controlled by a gate inside the pore of the channel (7–9). Electron crystallographic studies of nAChRs suggested that a leucine residue located in the middle of M2 region forms the gate of the channel (9, 10). Interestingly, this leucine is generally conserved in the ligand-gated channel superfamily and corresponds to position 317 of the GABA ρ1 subunit. Furthermore, we found that out of the eight positions in the M2 region that we mutated in these studies, mutations at only two positions, 317 and 314 (located one turn deeper in an α-helix toward the cytoplasmic end of the channel), result in spontaneous channel opening and reversal of agonist action (i.e., inhibition of this spontaneous channel opening by agonist). These two positions are located at or close to the region previously suggested to represent the channel gate, consistent with the notion that the observed effects may be due to changes in the properties of the channel gate (9, 10). Nonetheless, our results also indicate that even when both positions 314 and 317 are mutated the channel still manifests some gating activity. This finding implies that either the properties of the side-chains of the amino acid residues at the channel gate are not critical for the mechanism of gating or the concept of the "channel gate" may have to be broadened to include other regions of the channel pore. Of course, the possibility that the mutations we made result in a more global structural change cannot be totally excluded. It will remain for future experiments to elucidate the exact mechanism whereby the M2 mutants studied here influence channel gating and ligand regulation of channel gating.

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1. Noda, M., Takahashi, H., Tanabe, T., Toyosato, M., Kikyotani, S., Furutani, Y., Hirose, T., Takashima, H., Inayama, S., Miyata, T. & Numa, S. (1983) *Nature (London)* **302,** 528–532.
2. Grenningloh, G., Rienitz, A., Schmitt, B., Methfessel, C., Zensen, M., Beyreuther, K., Gundelfinger, E. D. & Betz, H. (1987) *Nature (London)* **328,** 215–220.
3. Maricq, A. V., Peterson, A. S., Brake, A. J., Myers, R. M. & Julius, D. (1991) *Science* **254,** 432–436.
4. Schofield, P. R., Darlison, M. G., Fujita, N., Burt, D. R., Stephenson, F. A., Rodriguez, H., Rhee, L. M., Ramachandran, J., Reale, V., Glencorse, T. A., Seeburg, P. H. & Barnard, E. A. (1987) *Nature (London)* **328,** 221–227.
5. Cutting, G. R., Lu, L., O'Hara, B. F., Kasch, L. M., Montrose-Rafizadeh, C., Donovan, D. M., Shimada, S., Antonarakis, S. E., Guggino, W. B., Uhl, G. R. & Kazazian, H. H., Jr. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 2673–2677.
6. Giraudat, J., Dennis, M., Heidmann, T., Chang, J. Y. & Changeux, J.-P. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 2719–2723.
7. Imoto, K., Busch. C., Sakmann, B., Mishina, M., Konno, T., Nakai, J., Bujo, H., Mori, Y., Fukuda, K. & Numa, S. (1988) *Nature (London)* **335,** 645–648.
8. Leonard, R. J., Labarca, C. G., Charnet, P., Davidson, N. & Lester, H. A. (1988) *Science* **242,** 1578–1581.
9. Unwin, N. (1993) *J. Mol. Biol.* **229,** 1101–1124.
10. Unwin, N. (1995) *Nature (London)* **373,** 37–43.
11. Monod, J., Wyman, J. & Changeux, J.-P. (1965) *J. Mol. Biol.* **12,** 88–118.
12. Karlin, A. (1967) *J. Theor. Biol.* **16,** 306–320.
13. Shimada, S., Cutting, G. & Uhl, G. R. (1992) *Mol. Pharmacol.* **41,** 683–687.
14. Feigenspan, A., Wässle, H. & Bormann, J. (1993) *Nature (London)* **361,** 159–162.
15. Qian, H. & Dowling, J. E. (1993) *Nature (London)* **361,** 162–164.
16. Zhang, D., Pan, Z.-H., Drideau, A. D., Zhang, X. & Lipton, S. A. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 11756–11760.
17. Gurusu, T. & Shingai, R. (1996) *Biochim. Biophys. Acta* **1305,** 15–18.
18. Xu, M. & Akabas, M. H. (1993) *J. Biol. Chem.* **268,** 21505–21508.
19. Xu, M., Covey, D. F. & Akabas, M. H. (1995) *Biophys. J.* **69,** 1858–1867.
20. Akabas, M. H., Kaufmann, C., Archdeacon, P. & Karlin, A. (1994) *Neuron* **13,** 919–927.
21. Woodward, R. M., Polenzani. L. & Miledi, R. (1993) *Mol. Pharmacol.* **43,** 609–625.
22. Pan, Z.-H. & Lipton, S. A. (1995) *J. Neurosci.* **15,** 2668–2679.
23. Galzi, J. L., Devillers-Thiery, A., Hussy, N., Bertrand, S., Changeux, J.-P. & Betrand, D. (1992) *Nature (London)* **359,** 500–505.
24. Betrand, D., Galzi, J. L., Devillers-Thiery, A. Bertrand, S. & Changeux, J.-P. (1993) *Proc. Natl. Acad. Sci USA* **90,** 6971–6975.
25. Katz, B. & Thesleff, S. (1957) *J. Physiol. (London)* **138,** 63–80.
26. Jackson, M. B. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 3901–3904.
27. Labarca, C., Nowak, M. W., Zhang, H., Tang, L., Deshpande, P. & Lester, H. (1995) *Nature (London)* **376,** 514–516.
28. Ohno, K., Hutchinson, D. O., Milone, M., Brengman, J. M. Bouza, C., Sine, S. & Engel, A. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 758–762.
29. Jackson, M. B. (1995) *Trends Biochem. Sci.* **19,** 396–398.
30. Changeux, J.-P. & Edelstein, S. J. (1995) *Trends Biochem. Sci.* **19,** 398–399.
31. Galzi, J.-L., Edelstein, S. J. & Changeux, J.-P. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 1853–1858.
32. Revah, F., Betrand, D., Galzi, J. L., Devillers-Thiery, A., Mulle, C., Hussy, N., Bertrand, S., Ballivet, M. & Changeux, J.-P. (1991) *Nature (London)* **353,** 846–849.
33. Yakel, J. L., Lagrutta, A., Adelman, J. P. & North, R. A. (1993) *Proc. Natl. Acad. Sci. USA* **93,** 1853–1858.
34. Filatov, G. N. & White, M. M. (1995) *Mol. Pharmacol.* **48,** 379–384.
35. Zukin, S. R., Young, A. B. & Snyder, S. H. (1974) *Proc. Natl. Acad. Sci. USA* **71,** 4802–4807.
36. Neubig, R. R. & Cohen, J. B. (1979) *Biochemistry* **18,** 5464–5475.
37. Jackson, M. B. (1988) *J. Physiol. (London)* **397,** 555–583.
38. Sine, S. M., Claudio, T. & Sigworth, F. J. (1990) *J. Gen. Physiol.* **96,** 395–437.