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Substitutions of the Highly Conserved M2 Leucine Create Spontaneously Opening ρ1 γ-Aminobutyric Acid Receptors

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ABSTRACT

All members of the receptor-operated ion channel family that includes γ-aminobutyric acid (GABA), glycine, nicotinic acetylcholine, and serotonin type 3 receptors have a conserved leucine near the center of the presumed second membrane-spanning domain. This leucine has been postulated to play a role in the gating of the pore. In this study, we examined the effects of mutating this leucine (L301) on the function of human homomeric ρ1 GABA receptors. Oocytes expressing ρ1 GABA receptors in which this leucine was substituted with alanine (A), glycine (G), serine (S), threonine (T), valine, or tyrosine, but not isoleucine or phenylalanine, demonstrated larger-than-normal resting conductances in the absence of GABA. This resting conductance had a reversal potential (and shifted reversal potential with chloride substitution) indistinguishable from that of the wild-type ρ1 GABA-activated current. This resting conductance was antagonized by picrotoxin and, in the case of the A, G, S, and T substitutions, by GABA itself. Although the ρ1 competitive antagonist 3-aminopropyl(methyl)-phosphinic acid did not block the resting conductance, this compound did competitively inhibit the GABA-mediated antagonism of the resting conductance. At higher concentrations, both 3-aminopropyl(methyl)-phosphinic acid and GABA directly activated the A, G, S, and T mutant receptors. Taken together, these data suggest that substitution of this highly conserved leucine with either small or polar residues produced ρ1 GABA receptors that can open in the absence of GABA and support the hypothesis that this leucine may play a key role in the gating of the pore.

GABA, glycine, nACh and 5-HT_3 receptors are members of a family of ligand-activated ion channels that mediate rapid neurotransmission in the mammalian central nervous system. These receptors are presumed to be pentamers (Langosch *et al*., 1988; Cooper *et al*., 1991; Nayeem *et al*., 1994) composed of subunits that each span the membrane four times (Noda *et al*., 1983; Grenningloh *et al*., 1987; Schofield *et al*., 1987; Maricq *et al*., 1991) with M2 of each subunit lining the pore (Leonard *et al*., 1988; Revah *et al*., 1991; Karlin and Akabas, 1995; Xu and Akabas, 1996). Based on an electron microscopic comparison of the nACh receptor in the closed and open states, it has been postulated that the pore is maintained in the closed position by hydrophobic interactions between the conserved leucines located near the center of the kinked helical M2 motifs (Unwin, 1995).

This invariant M2 leucine residue first was mutated in the homomeric α7 neuronal nACh receptor, where an increase was observed in sensitivity of the receptor to activation by ACh (Revah *et al*., 1991). In addition, a new single-channel conductance state appeared at low ACh concentrations, leading the authors to speculate that the mutation may have rendered the high affinity, nonconducting desensitized state ion conductive. An increase in agonist sensitivity also was observed when this leucine was mutated in the 5-HT_3 (Yakel *et al*., 1993), muscle nACh (Filatov and White, 1995; Labarca *et al*., 1995), and $GABA_A$ (Chang *et al*., 1996) receptors.

The ρ1 GABA receptor subunit first was cloned from the human retina (Cutting *et al*., 1991). Expression of this subunit produced homomeric GABA receptors with pharmacological and activation properties distinct from those of typical heteromeric $GABA_A$ receptors (Cutting *et al*., 1991; Amin and Weiss, 1994) and more like those of $GABA_C$ receptors (Johnston, 1986; Sivilotti and Nistri, 1989). For example, the EC_{50} value for GABA-mediated activation in ρ1 receptors is ≈40-fold lower that than in α1β2γ2 GABA receptors and the Hill coefficient is significantly greater (Kusama *et al*., 1993; Amin and Weiss, 1994) reflecting, at least in part, an increase in the number of agonist molecules required to gate the pore (Amin and Weiss, 1996). Moreover, ρ1 GABA receptors demonstrate very little desensitization during a maintained application of high concentrations of GABA (Amin and Weiss, 1994). Motivated by these unique ρ1 features, we set out to examine the potential role the conserved M2 leucine residue plays in the activation of homomeric ρ1 GABA receptors.

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ABBREVIATIONS: GABA, γ-aminobutyric acid; 3-APMPA, 3-aminopropyl(methyl)phosphinic acid; HEPES, 4-(2-hydroxyethyl)-1 piperazineethanesulfonic acid; M2, second membrane spanning domain; ACh, acetylcholine; nACh, nicotinic acetylcholine; 5-HT, 5-hydroxytryptamine.

A series of amino acids differing in their relative size and hydrophobicity were substituted for the conserved M2 leucine (L301). In contrast to the results in other members of this receptor-operated superfamily, these mutations did not increase the agonist sensitivity of homomeric ρ1 receptors. Instead, substitution with either polar or small residues produced large resting conductances in oocytes expressing these mutant receptors. Ionic substitution studies and the effects of GABA receptor agonists and antagonists indicated that the large resting conductance resulted from the spontaneous opening of the mutant GABA receptors. The observation that mutation of this leucine can destabilize the closed state of the pore is consistent with the hypothesis that this residue may play a key role in the gating of the pore.

Materials and Methods

Site-directed mutagenesis and *in vitro* transcription. The human ρ1 cDNA [obtained by the polymerase chain reaction (Amin and Weiss, 1994)] was cloned into the pALTER-1 vector (Promega, Madison, WI), and oligonucleotide-mediated site-directed mutagenesis was achieved with the Altered Sites Kit (Promega). Successful mutagenesis was verified by sequencing (Sanger *et al*., 1977).

cDNAs were linearized with *Nhe*I, which leaves a several-hundred-base pair tail. Run-off capped cRNA then was transcribed from the linearized cDNAs using standard *in vitro* transcription procedures as described in the Megascript *in vitro* transcription instruction manual (Ambion, Austin, TX). Integrity, as well as yield, of the cRNA was verified on an agarose gel.

Oocyte isolation and cRNA injection. *Xenopus laevis* (Xenopus I, Ann Arbor, MI) were anesthetized by hypothermia, and oocytes were surgically removed from the frog and placed in a solution that consisted of 82.5 mM NaCl, 2.5 mM KCl, 5 mM HEPES, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM Na_2HPO_4, 50 units/ml penicillin, and 50 μg/ml streptomycin, pH 7.5. Oocytes were dispersed in this solution minus Ca^{2+} plus 0.3% Collagenase A (Boehringer-Mannheim Biochemicals, Indianapolis, IN). After isolation, stage VI oocytes were thoroughly rinsed and maintained at 18°.

Micropipettes for injecting cRNA were pulled on a Sutter P87 horizontal puller, and the tips were cut off with the use of microscissors. cRNAs were diluted 2–20- fold with diethyl pyrocarbonate-treated water (depending on the yield of the *in vitro* transcription reaction). The cRNA was drawn up into the micropipette with negative pressure. To account for the batch-to-batch oocyte variability in expression level, we varied the injection volume such that 1–10 ng of cRNA of each construct was injected into a group of oocytes. In this way, we were ensured of having oocytes with the proper expression level for recording (typically 50–1000 nA maximum current). The cRNA was injected into the oocytes with a Nanoject delivery system (Drummond Scientific, Broomall, PA). We used the oocyte expression system because the long, stable recording times necessary to procure extensive dose-response relationships were not readily obtainable during patch-clamping of mammalian cells. Furthermore, preliminary studies indicate that high expression of the spontaneously opening channels seems to compromise the health of the human embryonic kidney 293 cells, and the expression level cannot be easily controlled when transfecting cDNA into mammalian cells. In contrast, we can easily control the expression level of recombinant proteins in oocytes by simply varying the amount of injected cRNA. By diluting the cRNA, as described above, we were able to keep the expression level of the mutant receptors in oocytes relatively low.

Recording. One to 3 days after injection, oocytes were placed on a 300-μm nylon mesh suspended in a small volume chamber (<100 μl). The oocyte was perfused continuously at a rate of 150–200 μl/sec with a solution consisting of 92.5 mM NaCl, 2.5 mM KCl, 5 mM HEPES, 1 mM $CaCl_2$, and 1 mM $MgCl_2$, pH 7.5, and briefly switched to the test solution that consisted of this same perfusion solution plus drug (e.g., GABA, GABA plus picrotoxin). For the chloride replacement experiments, 60 mM of the sodium chloride was replaced by 60 mM sodium isethionate. 3-APMPA was obtained from Tocris Cookson (St. Louis, MO). Picrotoxin was obtained from Sigma Chemical (St. Louis, MO).

Recording microelectrodes were fabricated with a P87 Sutter horizontal puller and filled with 3 M KCl. The electrodes had resistances of 1–3 MΩ. Standard two-electrode voltage-clamp techniques (GeneClamp 500; Axon Instruments, Foster City, CA) were used to record currents in response to application of agonist. Except when determining the current-voltage relationship, the membrane potential was clamped at −70 mV. Data were prefiltered at 10 Hz and played out on a Gould (Cleveland, OH) EasyGraf chart recorder during the experiment. The data also were digitized on-line using a Macintosh computer (Apple Computer, Cupertino, CA) equipped with a GW Instruments Data Acquisition Board (Somerville, MA). Digitization was carried out at 20 Hz using the software package Igor (Wavemetrics, Lake Oswego, OR) in conjunction with a set of macros written to drive the GW board (Bob Wyttenbach, Cornell University, Ithaca, NY). In addition, the currents were recorded on tape using the VR10B (Instrutech, Great Neck, NY) for off-line analysis.

The reversal potential of wild-type ρ1 GABA-mediated receptors presented in Fig. 1C and Table 1 was determined by varying the membrane potential during a continuous application of 3 μM GABA. This method more closely resembled the determination of reversal potentials in the spontaneously open mutant GABA receptors in that other membrane conductances contribute to the observed reversal potential. For example, in the chloride substitution experiments, the reversal potential determined using discrete pulses of GABA with the oocyte clamped at various membrane potentials was shifted positive by 26.5 ± 0.3 mV (predicted change in the Cl^- equilibrium potential was 30 mV) compared with a shift of 14.1 ± 4.9 mV determined by varying the membrane potential during continuous GABA application. All the experimental observations in this study were repeated in at least two separate batches of oocytes.

Data analysis. To quantify the agonist or antagonist sensitivity, each dose-response relationship was fit with one of the equations using a nonlinear least-squares method:

$$\text{Activation:}\quad I = \frac{I_{max}}{1 + (EC_{50}/[A])^n} \tag{1}$$

$$\text{Inhibition:}\quad I = \frac{I_{max}}{1 + ([A]/IC_{50})^n} \tag{2}$$

where I is the peak current at a given concentration of drug A; I_{max} is the maximum current response; EC_{50} or IC_{50} is the concentration of the drug yielding a half-maximal activation or inhibition of the GABA-activated current, respectively; and n is the Hill coefficient. The Kriskal-Wallis H test followed by a *post hoc* Mann-Whitney *U* test was used for statistical comparisons between wild-type and mutant parameters.

Results

Expression of ρ1 receptors with mutations at L301 resulted in unusually large resting currents in oocytes. As shown in Fig. 1A, oocytes expressing ρ1L301A, ρ1L301G, ρ1L301S, ρ1L301T, ρ1L301V, and ρ1L301Y, exhibited unusually large holding currents when the membrane potential was voltage clamped at −70 mV. The holding current at −70 mV in oocytes expressing ρ1L301F and ρ1L301I mutant receptors was not significantly different from that of wild-type receptors (Fig. 1A). Evidence that the increased holding currents resulted from the exogenously expressed mutant GABA receptors is provided in Fig. 1B; there was a

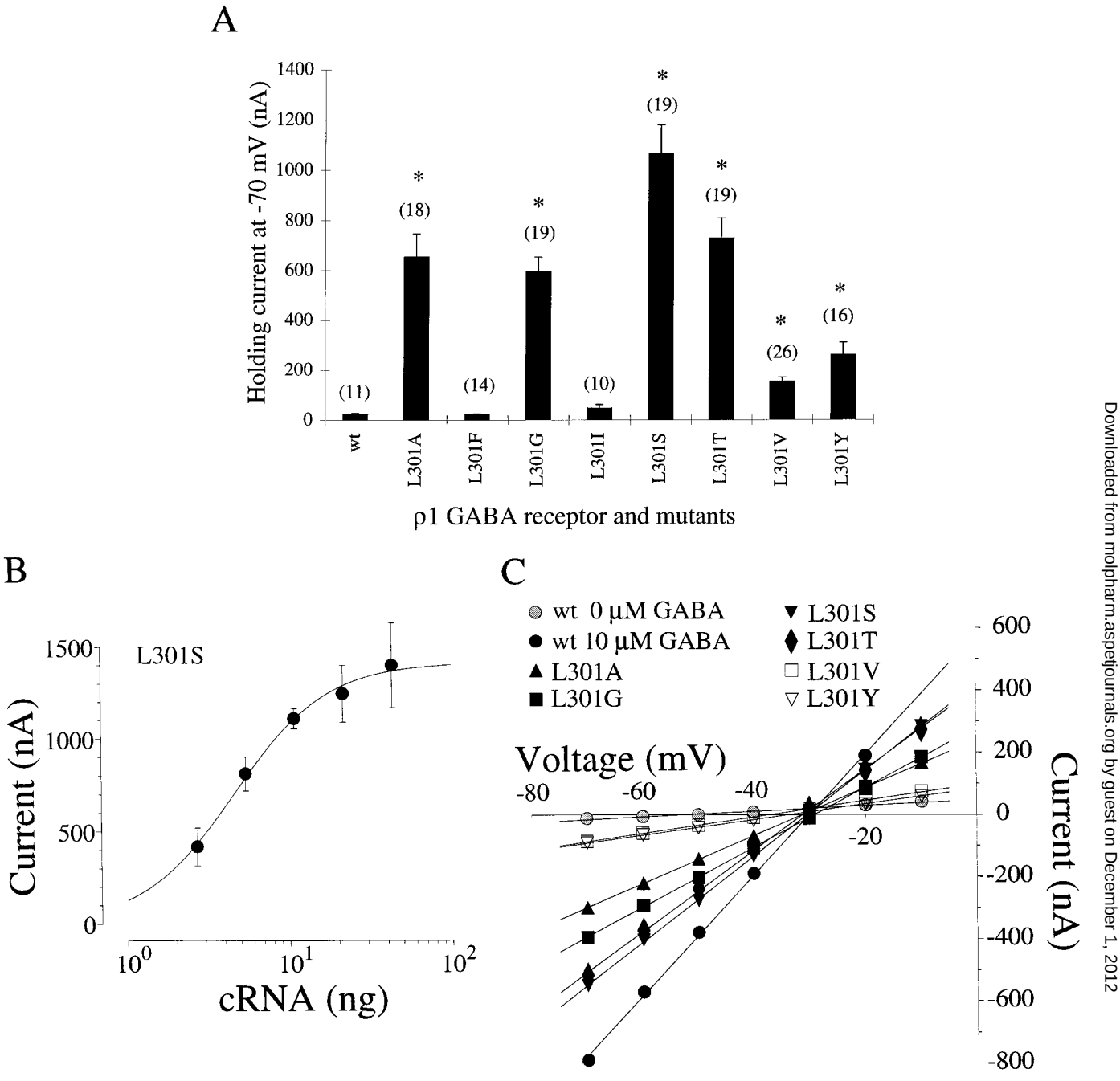

Fig. 1. Oocytes expressing mutant ρ1 GABA receptors have unusually large resting conductances. A, ρ1 receptors with substitutions of the conserved M2 leucine (L301) were expressed in *Xenopus laevis* oocytes. One to 3 days after cRNA injection, the current required to clamp the oocytes at −70 mV was measured. Substitution with either A, G, S, T, V, or Y required a significantly larger holding current than that of the wild-type receptor (*wt*). In contrast, the holding current for oocytes expressing either L301F or L301I mutants was not significantly different from that of the wild-type receptor. *Values above the bars*, number of oocytes for each case (mean ± standard error). B, Oocytes were injected with ρ1L301S cRNA, and 21 hr after injection, the holding current at −70 mV was measured using the two-electrode voltage-clamp. The average leakage current from 11 oocytes from the same batch were subtracted from the holding currents before plotting. Each point is the mean ± standard error from four to eight oocytes. *Continuous line*, least-squares fit of eq. 1 (see Materials and Methods) to the data points. Note that the holding current required to voltage clamp the oocytes at −70 mV increased as a function of the injected ρ1L301S cRNA. C, Current-voltage relationship of wild-type ρ1 GABA receptors in the absence of GABA (◍), wild-type ρ1 GABA receptors in the presence of 10 μM GABA (●), and L301 mutants in the absence of GABA (*all other filled symbols*). Current values for the wild-type receptor in the absence of GABA and all the mutants were determined by clamping the oocyte at the indicated membrane potential and measuring the holding current. Thus, the determined reversal potential is influenced by other membrane conductances. To mimic this situation in the wild-type ρ1 receptor, GABA was applied continuously, and the same procedure as described above was carried out to determine the current-voltage relationship (see Materials and Methods). *Continuous lines*, linear regression to the data points. Reversal potentials are provided in Table 1.

TABLE 1

Reversal potential and shift in reversal potential for wild-type and mutant ρ1 GABA receptors

The shift in reversal potential was determined by reducing the external chloride concentration from 99 to 39 mM. Values are mean ± standard deviation.

Combination	Reversal potential[a]	Reversal potential shift	Oocytes
	mV		*n*
ρ1	−53.7 ± 6.5	−2.4 ± 1.7	9
ρ1 (w/GABA)	−29.7 ± 3.9	14.1 ± 4.9	9
ρ1L301A	−31.3 ± 2.0	14.5 ± 3.9	6
ρ1L301G	−31.5 ± 2.3	13.2 ± 0.8	6
ρ1L301S	−30.1 ± 1.6	15.3 ± 2.4	6
ρ1L301T	−29.3 ± 2.3	12.7 ± 4.1	6
ρ1L301V	−33.5 ± 4.3	8.2 ± 1.9	6
ρ1L301Y	−31.0 ± 1.6	10.5 ± 1.0	6

[a] Reversal potential of the wild-type receptor was determined during a steady application of 10 μM GABA as described in the text.

dose-dependent increase in the holding current with increasing amounts of injected ρ1L301S cRNA. In >200 mutations we introduced into GABA receptors to date, many of which are in the M2, we never observed a large holding current as was evident with mutation of this conserved leucine.

We first considered the possibility that the large resting conductance was due to spontaneous openings of the mutant GABA receptors (i.e., opening in the absence of GABA). The data in Fig. 1C and Table 1 demonstrate that the reversal potential for this large resting conductance was the same as that of wild-type ρ1 GABA-mediated responses. Fig. 1C shows representative current-voltage relationships from oocytes expressing wild-type ρ1 receptors in the absence of GABA, wild-type ρ1 receptors during the continuous application of 10 μM GABA, and the mutants ρ1L301A, ρ1L301G, ρ1L301S, ρ1L301T, ρ1L301V, and ρ1L301Y in the absence of GABA. The reversal potential of the resting conductance in oocytes expressing wild-type ρ1 receptors in the absence of GABA was −53.7 ± 6.5 mV. The resting conductance in the mutant receptors demonstrated a reversal potential that was approximately −31 mV (range, −29 to −33 mV; Table 1), which was similar to the wild-type ρ1 reversal potential obtained during a continuous application of 10 μM GABA (−29.7 ± 3.9 mV).

To examine further the ionic basis of this large resting conductance, the external chloride concentration was reduced from 99 to 39 mM. This change in the chloride concentration should shift the chloride equilibrium potential positive by ≈30 mV. With chloride substitution, oocytes expressing wild-type ρ1 receptors exhibited only a very minimal shift in the reversal potential in the absence of GABA (Table 1), indicating that the oocyte native resting chloride conductance was minimal. In contrast, the reversal potential of the resting conductance for ρ1L301A, ρ1L301G, ρ1L301S, and ρ1L301T mutant receptors was shifted more positive by ≈14 mV (range, 13–15 mV). This shift is significantly less than the predicted 30-mV shift, most likely due to the contribution of other membrane conductances to the observed reversal potential (see Materials and Methods). In support of this, the wild-type reversal potential determined in the continuous presence of 10 μM GABA was shifted positive by a similar degree as the A, G, S, and T mutants (14.1 ± 4.9 mV). (The shift in reversal potential determined with pulses of GABA with the oocyte clamped at a range of membrane potentials was 26.5 ± 0.3 mV, much closer to the predicted shift for a chloride conductance.) The ρ1L301V and ρ1L301Y mutants demonstrated less of a shift in reversal potential with chloride substitution (Table 1), most likely due to the smaller resting conductance and therefore larger relative contribution of other native resting membrane conductances.

The data in Fig. 1 and Table 1 indicate that oocytes expressing ρ1L301A, ρ1L301G, ρ1L301S, ρ1L301T, ρ1L301V, and ρ1L301Y mutant receptors exhibit an increased conductance to chloride. We show that this large resting conductance is affected by selective GABA receptor agonists and antagonists that coupled with the data presented in Fig. 1 indicate that these particular substitutions produce spontaneously opening GABA receptors.

Picrotoxin reduced the large resting current in mutant ρ1 receptors. If the large resting conductances in oocytes expressing the ρ1L301A, ρ1L301G, ρ1L301S, ρ1L301T, ρ1L301V, and ρ1L301Y mutant receptors were due to spontaneous openings of the pore, they likely would be blocked by noncompetitive antagonists such as picrotoxin. Fig. 2A shows responses to a range of picrotoxin concentrations in an oocyte

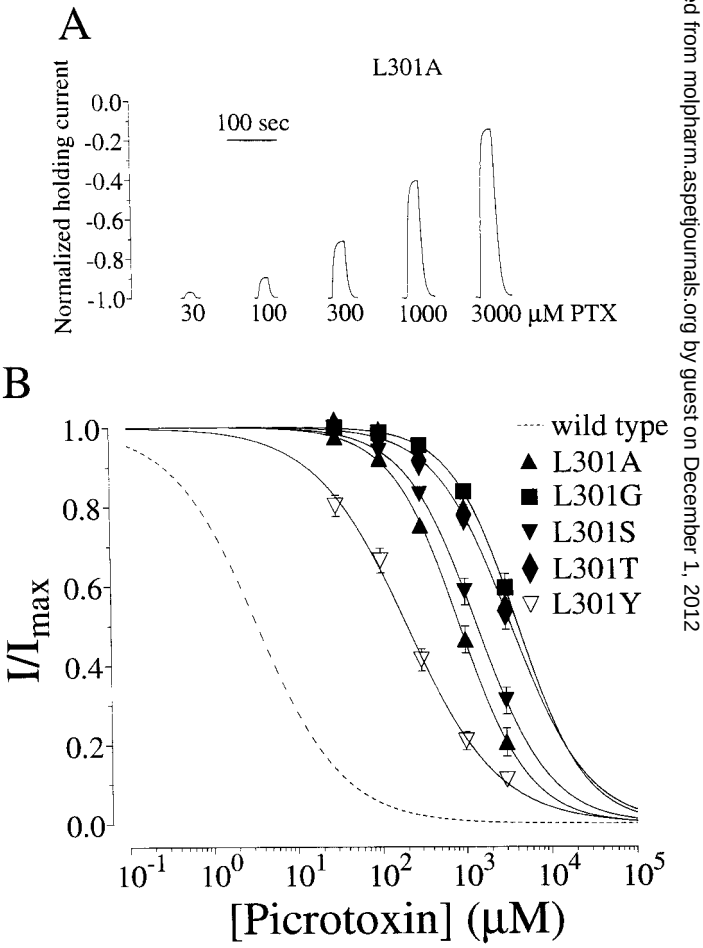

Fig. 2. Picrotoxin (*PTX*) blocks the large holding current in mutant ρ1 receptors. A, An oocyte expressing ρ1L301A receptors was exposed to increasing concentrations of picrotoxin. Picrotoxin blocked the holding current in a dose-dependent manner. The large holding current was normalized to −1.0. B, The percentage block is plotted as a function of the picrotoxin concentration for the A, G, S, T, and Y mutants. The data were fit with an inhibition function (eq. 2) assuming complete block by picrotoxin. Parameters from this fit are provided in Table 2. *Dashed line*, fit to data from wild-type ρ1 receptors activated by 3 μM GABA. Note that the mutation impaired sensitivity to picrotoxin. Values are mean ± standard error.

expressing ρ1L301A mutant receptors. (In Fig. 2A, the large resting current has been normalized to −1.0.) The application of picrotoxin reduced, in a concentration-dependent manner, the large inward current observed at −70 mV. Fig. 2B plots the fractional inhibition of the resting current as a function of the picrotoxin concentration for the ρ1L301A, ρ1L301G, ρ1L301S, ρ1L301T, and ρ1L301Y mutants. The ρ1L301V mutant exhibited no antagonism at concentrations as high as 3000 μM picrotoxin (data not shown). The application of these high concentrations of picrotoxin (3000 μM) did not antagonize the resting chloride conductance of oocytes expressing wild-type receptors (data not shown). The continuous lines are the best fits of eq. 2 to the data points (parameters are provided in Table 2). For purposes of comparison, the picrotoxin sensitivity of the wild-type ρ1 receptor (in the presence of 3 μM GABA) also is shown (*dashed line*).

In comparison to the wild-type ρ1 receptor, much higher concentrations of picrotoxin were required to block the mutants. Thus, substitution of the leucine at position 301 impaired picrotoxin sensitivity. This is not entirely unexpected as several studies have demonstrated that mutations in the M2 motif can impair the antagonism of GABA or glycine receptors by picrotoxin (Pribilla *et al.*, 1992; Zhang *et al.*, 1994, 1995; Enz and Bormann, 1995; Gurley *et al.*, 1995; Xu *et al.*, 1995). Nevertheless, the ability of picrotoxin to block this large resting conductance further supports the conclusion that the L301 mutations produce spontaneously opening GABA receptors.

Mutant receptors that open in response to GABA. Currents in response to a range of GABA concentrations were examined in oocytes expressing wild-type and L301 mutant ρ1 receptors. Through these experiments, the mutants were divided into two categories. The first category, shown in Fig. 3, was composed of receptors that demonstrated inward currents in response to GABA. Fig. 3A shows GABA-mediated currents from an oocyte expressing wild-type ρ1 receptors and GABA-mediated currents for the ρ1L301F, ρ1L301I, ρ1L301V, and ρ1L301Y mutant receptors. Although the sensitivity of the mutants to GABA seemed to be similar to that of the wild-type receptor, the waveform of the currents clearly were altered. In addition, the L301V mutant demonstrated an initial outward current in response to GABA, which was most obvious at the lower concentrations (e.g., 0.03 and 0.1 μM GABA). This likely is a GABA-mediated antagonism of the spontaneously opening receptors that also was evident in several of the other mutants. Fig. 3B shows average GABA dose-response relationships. The lines representing wild-type and mutants are from the best fits of the Hill equation (eq. 1) to these data. The EC_{50} values for the ρ1L301F, ρ1L301I, ρ1L301V, and ρ1L301Y mutant receptors were similar to that of the wild-type receptor (Table 3), although the slope of the dose-response relationship was decreased significantly (see Hill coefficients in Table 3).

In addition to the decrease in the Hill coefficient, these mutations altered the waveform of the GABA-mediated currents (Fig. 3C). Wild-type ρ1 GABA receptors expressed in oocytes demonstrated little desensitization during a prolonged pulse of GABA. In contrast, both ρ1L301F and ρ1L301Y mutant receptors exhibited a marked decline in current during GABA application, reminiscent of desensitization. Although the current through the ρ1L301I mutant receptors did not decline during the application of GABA, the rate at which the current decayed on GABA removal (deactivation) was reduced drastically. For the ρ1 wild-type receptor, the current dropped to 50% of the peak amplitude in 17.4 ± 1.3 sec (five experiments) on GABA removal compared with 190.8 ± 13.9 sec (five experiments) for ρ1L301I. Thus, although the L301F, L301I, and L301Y substitutions did not drastically impair the sensitivity of the receptor to GABA (e.g., no significant change in the EC_{50}), the gating kinetics of these mutants were altered (e.g., change in the Hill coefficient and current waveform).

Impaired picrotoxin sensitivity of mutant GABA receptors that are opened by GABA. Similar to the spontaneously opening A, G, S, T, V, and Y mutants, the ρ1L301I and ρ1L301F mutants exhibited an impaired picrotoxin sensitivity. Fig. 4A shows the effects of picrotoxin on wild-type ρ1 GABA receptors. The application of 3 μM GABA produced a large inward current that was blocked, in a concentration-dependent manner, by increasing concentrations of picrotoxin. Fig. 4A also shows the response of an oocyte expressing ρ1L301I receptors in response to the application of 1000 μM picrotoxin in the presence of 3 μM GABA. This extremely high concentration of picrotoxin blocked <10% of the GABA-activated current. Fig. 4B plots the fractional inhibition as a function of picrotoxin concentration for the wild-type, ρ1L301F, and ρ1L301I receptors. The phenylalanine substitution impaired picrotoxin sensitivity, although to a lesser extent than that of the isoleucine substitution. Fitting eq. 2 to these data yielded an IC_{50} value of 3.28 ± 0.43 μM and a Hill coefficient of 0.86 ± 0.03 for the ρ1 wild-type receptor and 119 ± 26 μM and 0.72 ± 0.04 for the IC_{50} value and Hill coefficient, respectively, of the ρ1L301F receptor. Due to the small amount of block, we could not reliably fit eq. 2 to the ρ1L301I data. Nevertheless, the ρ1L301F and ρ1L301I mutations induced ≈36- and >305-fold decreases in picrotoxin sensitivity compared with the wild-type receptor.

Mutant receptors that close in response to GABA. Fig. 5A shows current responses to the application of GABA for the second category of mutants: ρ1L301A, ρ1L301G, ρ1L301S, and ρ1L301T. Surprisingly, oocytes expressing these mutant receptors demonstrated a dose-dependent increase in an apparent outward current in response to GABA as opposed to the typical inward current observed in oocytes expressing wild-type ρ1 GABA receptors (Fig. 3). To test whether this apparent outward current was associated with an increase or a decrease in membrane conductance, we

TABLE 2

Sensitivity of mutant receptors to picrotoxin

All values are mean ± standard deviation. Picrotoxin-mediated antagonism of the ρ1 wild-type receptor, as well as the L301F and L301I mutants, were determined on currents activated by 3 μM GABA. Antagonism of the resting conductance was determined in the other mutants. The L301I mutation exhibited a 10% inhibition with 1000 μM picrotoxin, whereas the L301V mutation exhibited no antagonism at picrotoxin concentrations as high as 3000 μM.

Combination	IC_{50}	Hill coefficient	Oocytes
	μM		*n*
ρ1	3.28 ± 0.43	0.86 ± 0.03	6
ρ1L301A	970 ± 406	1.08 ± 0.10	6
ρ1L301F	119 ± 26	0.72 ± 0.04	6
ρ1L301G	4920 ± 1503	1.11 ± 0.15	6
ρ1L301I	N.D.	N.D.	6
ρ1L301S	1594 ± 573	1.02 ± 0.05	6
ρ1L301T	5454 ± 2987	0.95 ± 0.11	6
ρ1L301V	N.D.	N.D.	6
ρ1L301Y	192 ± 67	0.98 ± 0.07	6

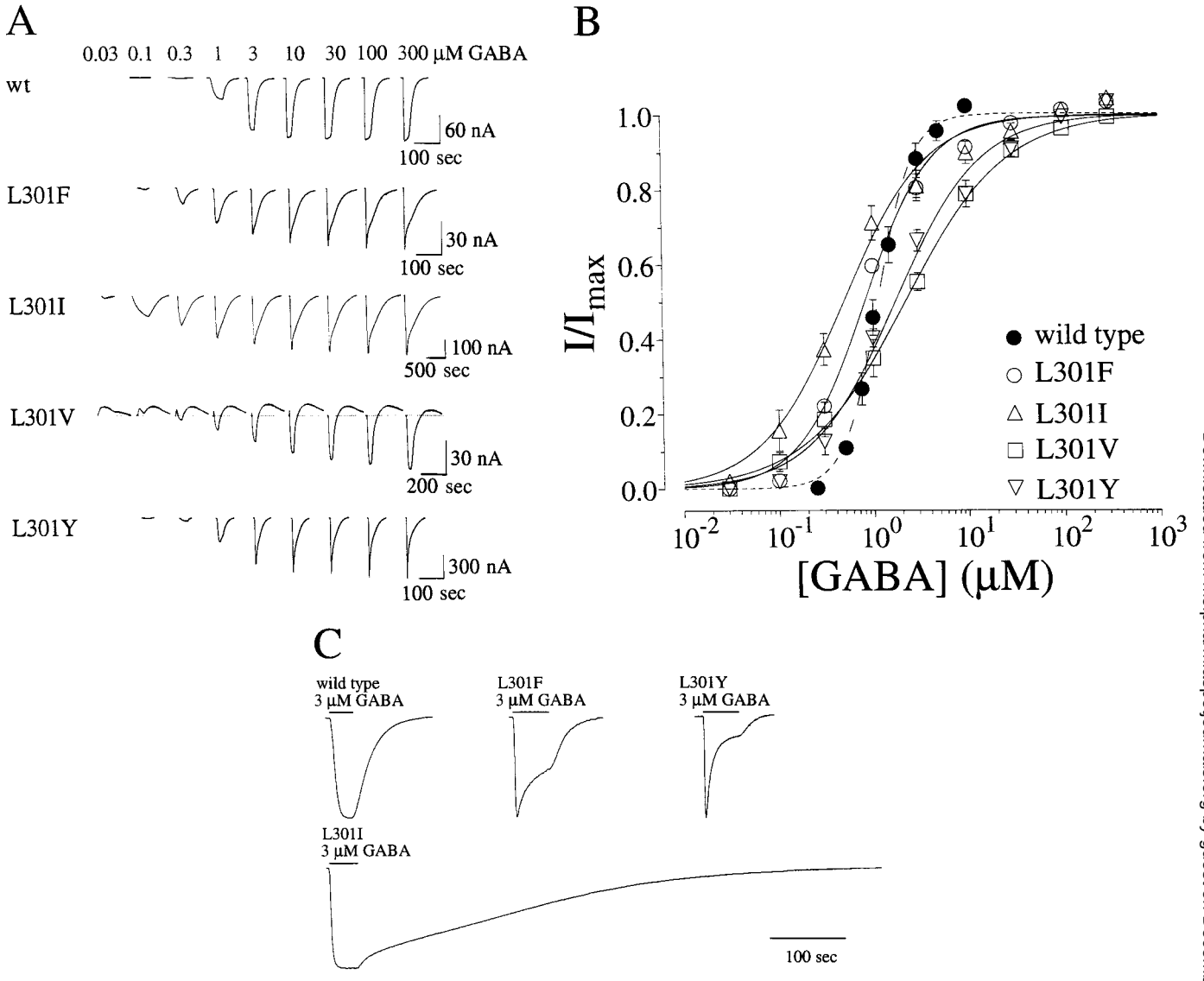

Fig. 3. GABA-mediated activation of wild-type (*wt*) ρ1L301F, ρ1L301I, ρ1L301V, and ρ1L301Y receptors. A, Currents in response to a range of GABA concentrations (*top*) are shown. Although the waveform of the currents vary between mutants (note different time scales), the mutant receptors have a GABA sensitivity similar to that of the wild-type receptor. Note that the L301V mutant shows an initial outward current most obvious at the lower GABA concentrations. This represents a GABA-mediated antagonism of spontaneously opening mutant receptors. B, Amplitude of the currents are plotted as a function of the GABA concentration. ●, Wild-type ρ1 receptor. *Dashed and continuous lines*, best fit of the Hill equation (eq. 1) to the data points. Parameters from the fits are provided in Table 3. Values are mean ± standard error. For the measurement of amplitudes in the L301V mutant, the base-line was the level before GABA application. C, Examples of GABA-activated currents (3 μM GABA) in wild-type, L301F, L301I, and L301Y receptors. The current through the wild-type receptors does not decay during the continuous application of 3 μM GABA. In contrast, GABA-activated currents from ρ1L301F and ρ1L301Y receptors exhibited a decay during agonist application reminiscent of desensitization. The ρ1L301I mutant did not decay during GABA application, but channel closure on agonist removal (deactivation) was slowed considerably ($t_{½}$ = 17.4 ± 1.3 sec for the wild-type and 190.8 ± 13.9 sec for ρ1L301I). An amplitude calibration bar is not shown because these currents were scaled to have the same peak.

applied hyperpolarizing voltage steps (−10 mV, 0.33 Hz) during the GABA application. Fig. 5B shows one representative experiment for the ρ1L301T mutant receptor. The application of GABA decreased the current step in response to the 10-mV voltage step, indicating a decrease in membrane conductance. Thus, the apparent outward currents in Fig. 5A actually were decreases in inward currents. Fig. 5C shows the dose dependence of this decrease in inward current for the L301A, L301G, L301S, and L301T mutants fit by eq. 2 (*continuous lines*). The parameters from these fits are provided in Table 4. The GABA IC_{50} values for the mutant receptors (range, 0.021–0.33 μM) were significantly lower than the EC_{50} value of the wild-type receptor (≈1 μM). Given that the ρ1L301A, ρ1L301G, ρ1L301S, and ρ1L301T mutants exhibited the unusually large, picrotoxin-blockable, resting chloride conductance (Figs. 1 and 2), our interpretation of the results in Fig. 5 is that the binding of GABA actually closes the spontaneously opening mutant GABA receptors. Possible mechanisms of this antagonism are considered in Discussion.

The competitive antagonist 3-APMPA inhibits the GABA-mediated antagonism of spontaneously opening mutant receptors. Fig. 6A confirms that 3-APMPA is a competitive antagonist of wild-type ρ1 GABA receptors (Ragozzino *et al.*, 1996). Represented in this figure are the wild-type GABA dose-response relationship in the absence of 3-APMPA and the GABA dose-response relationship, in the

TABLE 3
Activation of wild-type and mutant receptors by GABA
Parameters are mean ± standard deviation.

Combination	EC_{50}	Hill coefficient	Oocytes
	μM		n
ρ1	1.16 ± 0.26	2.36 ± 0.48	6
ρ1L301F	0.85 ± 0.14	1.26 ± 0.21	6
ρ1L301I	0.55 ± 0.23	1.13 ± 0.50	5
ρ1L301V	2.27 ± 0.89	0.87 ± 0.15	6
ρ1L301Y	1.81 ± 0.59	1.01 ± 0.30	6

same three oocytes, in the presence of 10 μM 3-APMPA. This concentration of 3-APMPA shifted the EC_{50} value from 1.0 ± 0.1 to 12.7 ± 1.0 μM with no significant change in the maximum current. The Hill coefficient was decreased from 2.71 ± 0.13 to 1.9 ± 0.01, suggesting, in the strict sense, the antagonism may not be purely competitive. Nevertheless, in terms of the current amplitude, the actions of 3-APMPA were totally surmountable.

Classically, competitive antagonists are presumed to exert their actions by preventing the binding of the ligand to the receptor (e.g., overlapping binding sites of agonist and antagonist). Given that the ρ1L301A, ρ1L301G, ρ1L301S, and ρ1L301T receptors can open without the binding of GABA, we expected 3-APMPA to not antagonize the spontaneously opening GABA receptors, and this was indeed the case (data not shown). Surprisingly, at higher concentrations than necessary to antagonize wild-type GABA-activated currents (≈1 μM) (Ragozzino *et al.*, 1996), the application of 3-APMPA evoked a further inward current on its own (Fig. 6, B and C). The expense of this compound precluded a thorough determination of the dose-response relationship. Clearly, the EC_{50} value for 3-APMPA-mediated activation was >10 μM. 3-APMPA did not activate the wild-type ρ1 receptor (data not shown).

Although 3-APMPA did not antagonize the spontaneously opening mutant receptors, this compound did antagonize the GABA-mediated antagonism. Fig. 7A shows the antagonism of L301A mutant receptors by GABA, similar to that presented in Fig. 5. Also shown are GABA-mediated responses in the same oocyte but in the presence of 3 μM APMPA. The small inward currents in the presence of 0.03 and 0.1 μM GABA plus 3 μM 3-APMPA are due to the activation by 3-APMPA, as documented in Fig. 6B. In the presence of 3-APMPA, higher concentrations of GABA were required to antagonize the receptors. The average dose-response relationship in the absence and presence of 3 μM 3-APMPA for three oocytes is plotted in Fig. 7B along with the best fit of eq. 2 to these data. The IC_{50} value and Hill coefficient in the absence of 3-APMPA were 0.12 ± 0.01 μM and 2.73 ± 0.31, respectively. The presence of 3 μM 3-APMPA shifted the inhibition curve to the right with an IC_{50} value for GABA of 0.64 ± 0.02 μM and a Hill coefficient of 2.43 ± 0.13. These data indicate that 3-APMPA competitively antagonized the GABA-mediated inhibition of these spontaneously opening receptors, supporting the notion that the site at which GABA binds to antagonize the mutant receptors is similar, if not the same, as that to which GABA binds to activate the wild-type receptor.

Effects of higher GABA concentrations on the spontaneously opening GABA receptors. Fig. 8A shows GABA-mediated currents for the ρ1L301A mutant receptor

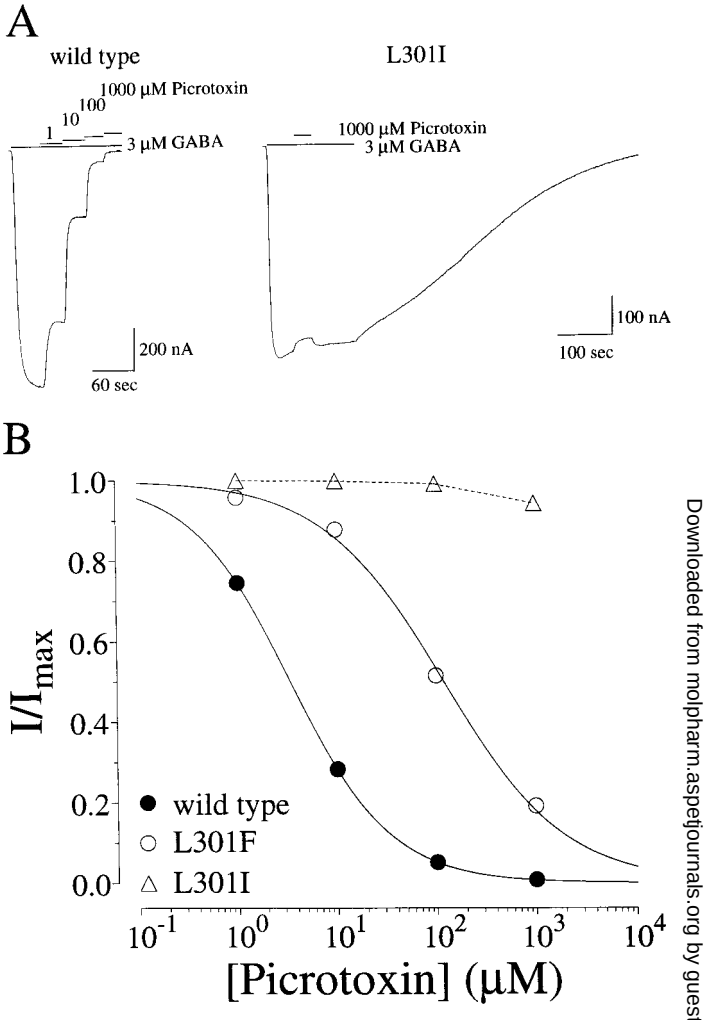

Fig. 4. Antagonism of ρ1L301F and ρ1L301I receptors by picrotoxin. A, *Trace on the left*, increasing concentrations of picrotoxin applied to an oocyte expressing wild-type ρ1 GABA receptors during the continuous application of 3 μM GABA. Picrotoxin produced a dose-dependent inhibition of the GABA-activated current. *Trace on the right*, application of 1000 μM picrotoxin during the application of 3 μM GABA to an oocyte expressing the ρ1L301I mutant. This high concentration of picrotoxin blocked only a small fraction of the GABA-activated current. B, Percentage block of the GABA-activated current is plotted against the picrotoxin concentration for wild-type, L301F, and L301I receptors. *Continuous lines*, best fit of the inhibition equation to these data (eq. 2). The parameters from this fit are provided in Table 2. *Dashed line* (L301I), just connects the points because we were unable to fit eq. 2 to these data due to the small amount of block.

and includes higher GABA concentrations than those presented in Fig. 5. Up to 1 μM, the GABA-mediated antagonism is observed, as documented in Fig. 5. As the GABA concentration exceeded 1 μM, a slowly developing inward current became apparent. We interpret the current trace at 1000 μM GABA (shown on an expanded time scale in the bottom of Fig. 8A) as follows. With GABA application, the spontaneously open GABA receptors first close producing a decreasing inward current. With a slower time course, the receptors open, producing the observed inward current. On the initiation of GABA removal, the channels first close as the GABA concentration falls and then reopen in the absence of GABA. Such multiphasic responses also were observed for the ρ1L301G, ρ1L301S, and ρ1L301T mutant receptors. Fig.

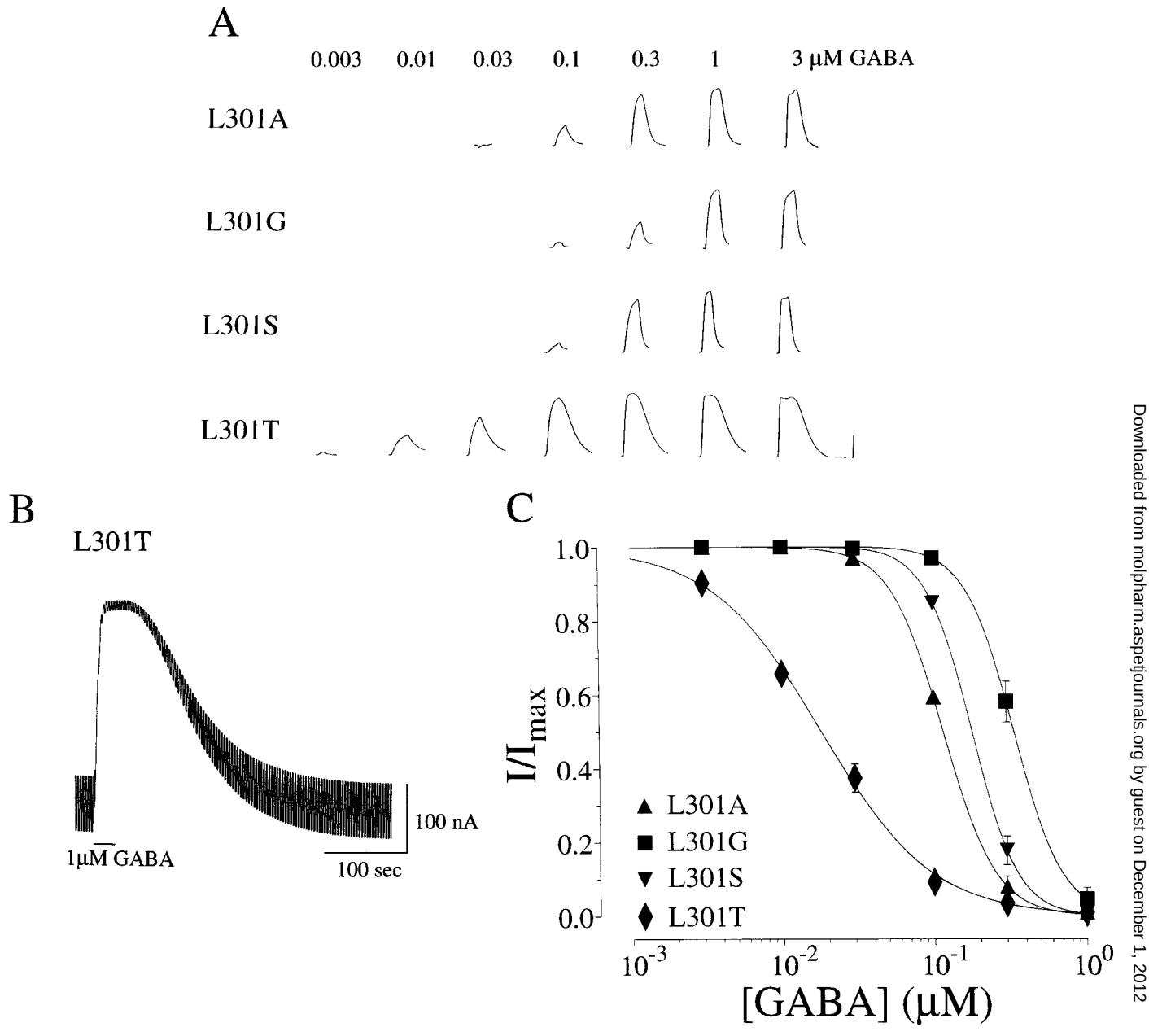

Fig. 5. GABA antagonizes the spontaneously opening GABA receptors. A, Currents in response to increasing concentrations of GABA were examined in oocytes expressing ρ1L301A, ρ1L301G, ρ1L301S, and ρ1L301T receptors. Note the concentration-dependent increase in an apparent outward current as opposed to the inward current expected at −70 mV. *Calibration bar*, 100 sec and 50 nA, 60 nA, 180 nA, and 150 nA, for the A, G, S, and T mutants, respectively. B, Hyperpolarizing voltage steps (10 mV) were applied to an oocyte expressing L301T receptors before, during, and after the application of 1 μM GABA. The current step in response to the voltage step decreased during the application of GABA, indicating a GABA-mediated decrease in conductance. Thus, the application of GABA closes ion channels. C, The fraction of current blocked is plotted against the GABA concentration and fitted by an inhibition equation (eq. 2, *continuous lines*). Values are mean ± standard error, and the parameters from the fits are provided in Table 4.

8B plots the steady state amplitude of the current for an individual oocyte over the entire GABA concentration range (see figure legend for details of amplitude measurements). The continuous lines are the best fit of eqs. 1 and 2 to the GABA-mediated activation and inhibition, respectively. The EC_{50} value for the GABA-mediated activation of the L301A mutant was 127 ± 72 μM with a Hill coefficient of 0.66 ± 0.04 (four experiments). Parameters for the GABA-mediated antagonism were presented in Fig. 5 and Table 4.

The rising phase of this inward current (Fig. 8A, *ii*), similar to the 3-APMPA-mediated current shown in Fig. 6B (at concentrations of >10 μM), was extremely slow and multiphasic. Fig. 8C shows the similarity in the activation kinetics of current responses in the same oocyte to 100 μM GABA or 100 μM 3-APMPA. These data indicate complex gating kinetics and a similar mechanism of activation for these two compounds. The observation that 3-APMPA can activate the mutant receptors suggests GABA and 3-APMPA have overlapping (if not super-

TABLE 4
Inhibition of spontaneous mutant receptors by GABA
Values are mean ± standard deviation.

Combination	IC_{50}	Hill coefficient	Oocytes
	μM		n
ρ1L301A	0.12 ± 0.01	2.90 ± 1.02	6
ρ1L301G	0.33 ± 0.06	3.83 ± 1.81	6
ρ1L301S	0.18 ± 0.03	3.15 ± 0.75	6
ρ1L301T	0.021 ± 0.005	1.32 ± 0.07	6

imposable) binding sites. This also is supported by the close resemblance of the structures of GABA and 3-APMPA (Ragozzino *et al.*, 1996).

Discussion

Comparison with other studies. This highly conserved M2 leucine residue was first mutated in the α7 neuronal nACh receptor, where a leftward shift in the ACh dose-response relationship was observed (Revah, *et al.*, 1991). The degree of shift in agonist sensitivity was dependent on the particular residue substituted at this position. In addition, the rate of desensitization was decreased and a new single-channel conductance state was observed at low ACh concentrations. The authors postulated that the mutation-induced increase in ACh sensitivity was the result of the desensitized state (which has a higher agonist affinity) becoming ion permeant. An increase in agonist sensitivity and rate of desensitization also was observed for mutation of the homologous leucine in 5-HT_3 (Yakel *et al.*, 1993), heteromeric muscle nACh (Filatov and White, 1995; Labarca *et al.*, 1995), and α1β2γ2 GABA receptors (Chang *et al.*, 1996).

There are some similarities and some differences between our results in the homomeric ρ1 GABA receptor and the results observed in these other ligand-activated receptors. With regard to desensitization, mutation of the conserved leucine altered the rate of current decay during prolonged agonist application for α7, 5-HT_3, and ρ1 receptors. However, there are key differences among the receptors regarding the particular residue substituted at this position and the observed effect. For example, all substitutions in the α7 receptor slowed the rate of current decay during a prolonged ACh application, with a decay rate order of L > F > V > T > S

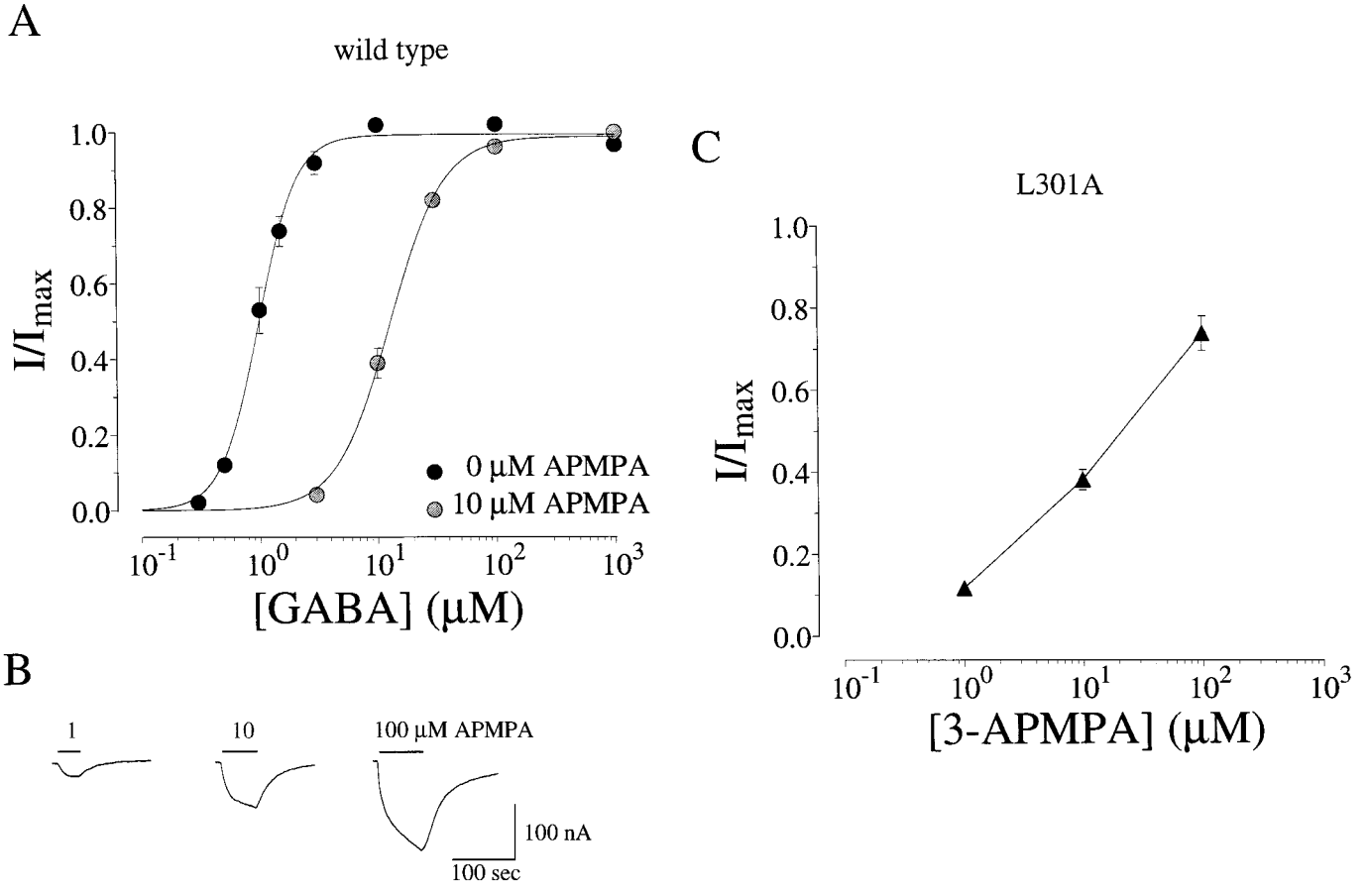

Fig. 6. Actions of the competitive antagonist 3-APMPA on wild-type and mutant GABA ρ1 receptors. A, GABA dose-response relationship in the absence (*filled symbols*) and presence (*stippled symbols*) of 10 μM 3-APMPA. *Continuous lines*, best fit of the Hill equation (eq. 1) to the data points. The EC_{50} value and Hill coefficient in the absence of 3-APMPA were 1.0 ± 0.1 μM and 2.71 ± 0.13, respectively. The presence of 3-APMPA (10 μM) shifted the EC_{50} value to 12.7 ± 1.0 μM, and the Hill coefficient was reduced to 1.9 ± 0.01. The amplitude of the GABA-mediated currents in the presence of 3-APMPA was normalized to the maximum current in the absence of 3-APMPA, indicating the actions of this compound were totally surmountable by GABA. B, At low concentrations, 3-APMPA did not antagonize the spontaneously opening mutants: L301A, L301G, L301S, and L301T (data not shown). However, as the concentration exceeded 1 μM, 3-APMPA evoked an inward current on its own. Currents are shown in response to 1, 10, and 100 μM 3-APMPA for an oocyte expressing ρ1L301A receptors. Note the multiphasic activation of the current most obvious at 10 and 100 μM 3-APMPA. C, Dose-response relationship for the 3-APMPA-mediated activation. Values are mean ± standard error. *Continuous line*, merely connects the points. The cost of this compound prohibited an examination of higher concentrations to quantify the 3-APMPA sensitivity (e.g., EC_{50} value).

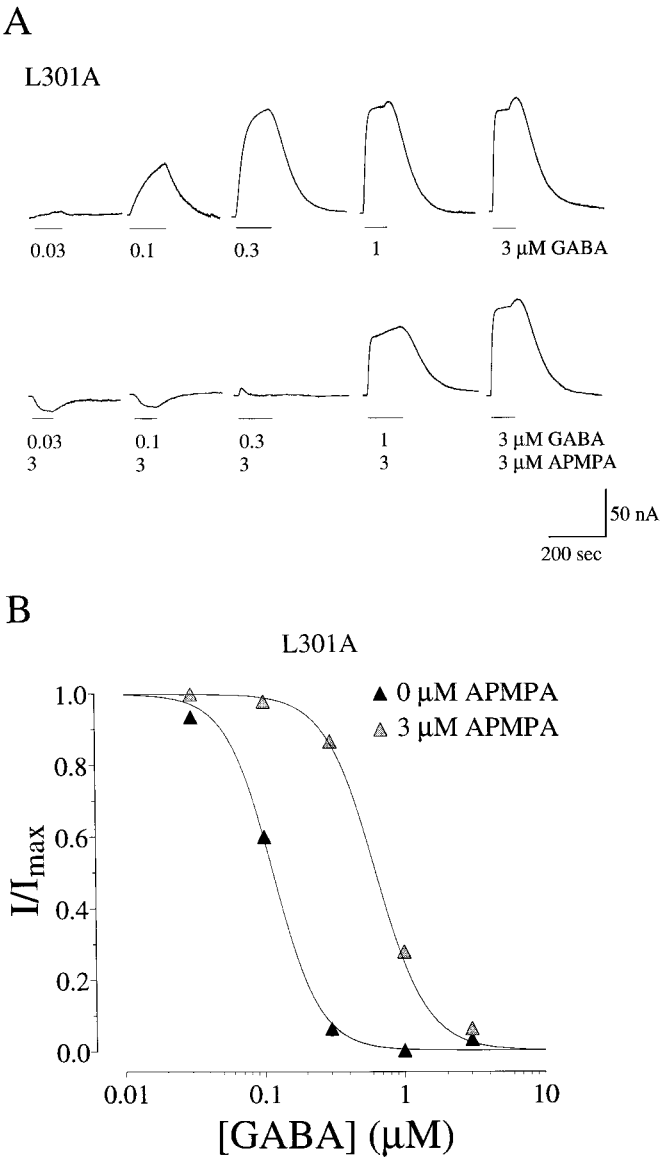

Fig. 7. 3-APMPA antagonizes the GABA-mediated antagonism of spontaneously opening mutant receptors. A, *Top row of traces*, GABA-mediated currents in oocytes expressing ρ1L301A receptors. This is the GABA-mediated antagonism presented in Fig. 5. *Bottom row of traces*, GABA-mediated currents from the same oocyte but in the presence of 3 μM 3-APMPA. The small inward currents at the two lower concentrations are the result of direct activation by 3-APMPA. 3-APMPA shifted the sensitivity of the GABA-mediated antagonism to the right. B, Dose-response relationship for the GABA-mediated antagonism of ρ1L301A receptors in the absence and presence of 3-APMPA (3 μM). *Continuous lines*, best fit of eq. 2 (Materials and Methods) to the data points (mean ± standard error, three experiments). *Error bars*, smaller than the plotted symbols. The IC_{50} value and Hill coefficient for the GABA-mediated antagonism in the absence of 3-APMPA were 0.12 ± 0.01 μM and 2.73 ± 0.31, respectively. The presence of 3-APMPA shifted the IC_{50} value for GABA to 0.64 ± 0.02 μM, and the Hill coefficient was 2.43 ± 0.13. The amplitude of the responses in the presence of 3-APMPA was normalized to the maximum in the absence of 3-APMPA.

(Revah *et al.*, 1991), a rough correlation with the degree of amino acid hydrophobicity of F ≈ V ≈ L > A > Y > T > S (Eisenberg *et al.*, 1982). In contrast, the desensitization rate in 5-HT_3 receptors was F > Y > A > L > T, which does not correlate well with the degree of hydrophobicity (Yakel *et al.*, 1993). GABA-activated currents in oocytes expressing wild-type ρ1 receptors do not decay during a maintained application of GABA (Amin and Weiss,1994), although the L301F and L301Y substitutions produced ρ1 GABA-mediated currents that did decay during continuous GABA application with a rate of the order Y > F > L. Because only two of the substitutions produced this desensitizing phenotype in ρ1 receptors, it is difficult to draw a conclusion on the degree of hydrophobicity at position 301 and the rate of current decay.

Nevertheless, it is clear from the current study, as well as the studies with α7 and 5-HT_3 (Revah *et al.*, 1991; Yakel *et al.*, 1993), that the particular residue at this position can affect receptor desensitization. The differences in the results among the ρ1, α7, and 5-HT_3 receptors noted above, along with the pitfall that any amino acid substitution will alter both the hydrophobicity and size of the side chain, prevent a correlation on the hydrophobicity of the amino acid at this position and the process of receptor desensitization. The recently developed technique for introducing unnatural amino acids, by providing a larger array of possible substitute residues, may facilitate such an endeavor (Kearney *et al.*, 1996). Furthermore, studies using small cells or membrane patches in which an agonist can be rapidly introduced and removed demonstrate that the current decay due to desensitization has multiple components (Verdoorn *et al.*, 1990). In these cases, some of the components are faster than can be resolved in the current study because it takes a longer period of time to apply and remove agonists to oocytes. These limitations further complicate a correlation of amino acid characteristics and the rate of desensitization.

With regard to activation, the leucine mutations in the homomeric α7 (Revah *et al.*, 1991) and 5-HT_3 (Yakel *et al.*, 1993) receptors demonstrated an increase in agonist sensitivity that correlated with the degree of hydrophobicity (i.e., the lower the hydrophobicity, the greater was the leftward shift in the EC_{50} value). Similarly, in heteromeric receptors, mutation of this highly conserved leucine in the muscle nACh (Kearney *et al.*, 1996) and $\alpha1\beta2\gamma2$ GABA receptors demonstrated an increase in agonist sensitivity with a substitution of the leucine in any one of the five subunits (Chang *et al.*, 1996). Our results are distinct from these studies in that substitution of this leucine did not induce a leftward shift in the agonist dose-response relationship but rather substitutions of hydrophilic (Y, S, and T) or small (G, A, and V) residues produced spontaneously opening receptors. (Although in the limiting sense, opening in the absence of agonist could be considered an increase in agonist sensitivity.) Other substitutions (F, Y, and V) that decreased the EC_{50} values of the α7 and 5-HT_3 receptors did not alter the EC_{50} value of the GABA ρ1 receptor, although the Hill coefficient was depressed, perhaps indicating an effect on the cooperativity of activation. For $\alpha1\beta2\gamma2$ GABA receptors, oocytes expressing the leucine-mutant receptors had an increased resting conductance compared with wild-type $\alpha1\beta2\gamma2$ GABA receptors, leading the authors to speculate that this might be due to spontaneous openings of the pore (Chang *et al.*, 1996). Tierney *et al.* (1996) demonstrated that substitution of this leucine in either the α1 or β1 subunit does induce spontaneously opening $\alpha1\beta1$ GABA receptors expressed in SF9 cells. In addition, substitution of this leucine in the α subunit of muscle nACh receptors (Auerbach *et al.*, 1996) increased the frequency of occurrence of spontaneous openings (Jackson, 1984). Finally, spontaneously opening GABA receptors have been reported with expression of bovine α2 subunits alone

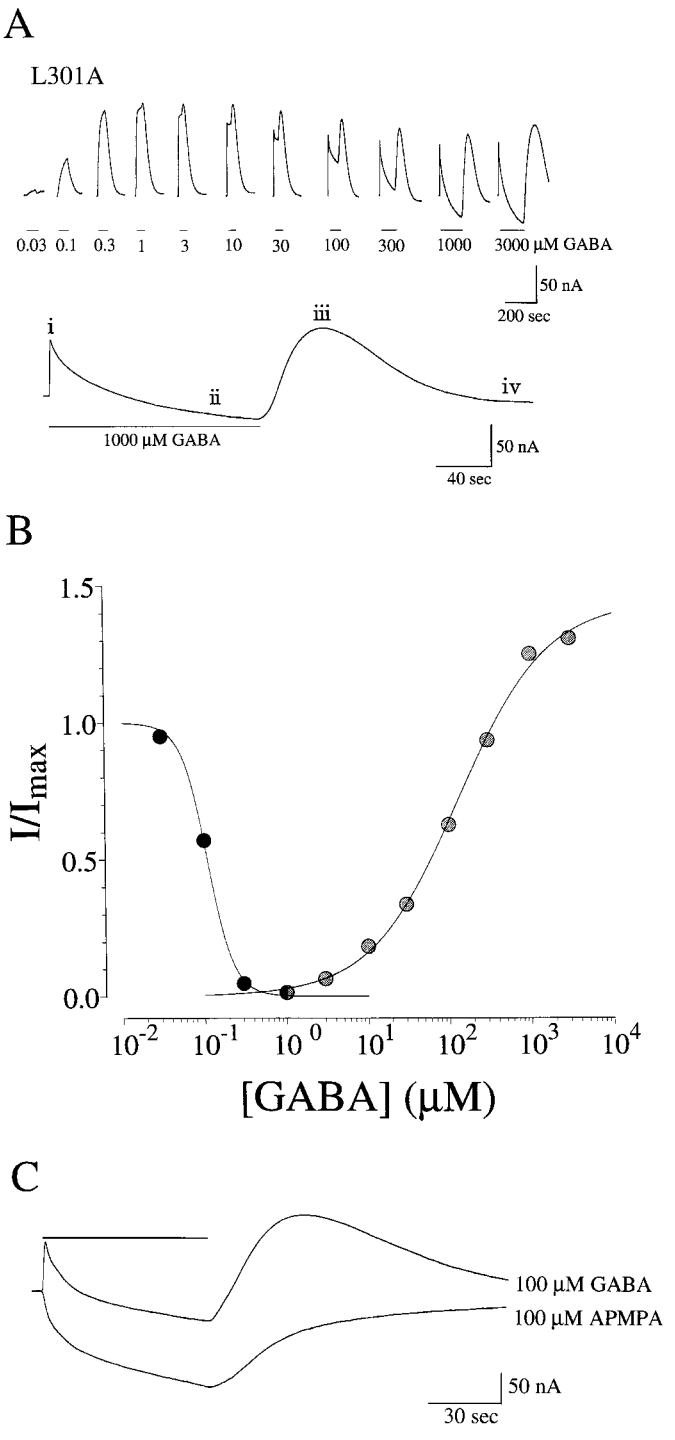

Fig. 8. At high concentrations, GABA activated the spontaneously opening mutant receptors. A, Currents in response to a wide range of GABA concentrations are shown from an oocyte expressing ρ1L301A mutant receptors. From 0.03 to 1 μM GABA, the antagonism of the spontaneously opening receptors was evident. At higher GABA concentrations, an increasing inward current became apparent. *Bottom*, current shows the response to 1000 μM GABA on an expanded time scale. The application of GABA initially blocked the receptors (*i*), but over a slower time course, the receptors were activated (*ii*). As the GABA concentration fell, the channels reclosed (*iii*) and finally reopened in the absence of GABA (*iv*). B, Complete dose-response relationship to GABA in an oocyte expressing ρ1L301A receptors. *Filled symbols*, GABA-mediated antagonism, and the base-line was the current level before GABA application. *Shaded symbols*, GABA-mediated activation, and the base-line was the extrapolated current level from the antagonism observed at low GABA concentrations. *Continuous lines*, best fits of eqs. 1 and 2 (see Materials and Methods) to the data points. The parameters from these fits are inhibition, IC_{50} = 0.11 μM with a Hill coefficient of 2.7; activation, EC_{50} = 132 with a Hill coefficient of 0.80. C, Currents in response to 100 μM GABA or 100 μM 3-APMPA from an oocyte expressing ρ1L301A mutant receptors. Note the similarity in the multiphasic activation kinetics.

(Blair *et al.*, 1988), rat β1 subunits alone (Sigel *et al.*, 1989), and a combination of rat α4 and β subunits (Khrestchatisky *et al.*, 1989).

Mutations of the conserved leucine also impaired the ability of picrotoxin to antagonize the ρ1 receptor. Several studies have demonstrated that mutations in the M2 motif can impair the antagonism of GABA or glycine receptors by picrotoxin (Pribilla *et al.*, 1992; Zhang *et al.*, 1994, 1995; Enz and Bormann, 1995; Gurley *et al.*, 1995; Xu *et al.*, 1995). This impairment differed dramatically among the various L301 mutants. For example, the IC_{50} value for picrotoxin was ≈119 μM for ρ1L301F and >5450 μM for ρ1L301T. There was no obvious correlation between the picrotoxin sensitivity and either the mutant phenotype (GABA activated as in Fig. 3 versus spontaneously opening as in Fig. 5) or the particular residue substituted at position 301. The observation that the particular substitution can have widely different effects on the picrotoxin sensitivity suggests this highly conserved leucine may be closely associated with the site of action of picrotoxin. Furthermore, as is true for several other residues postulated to be crucial for the actions of picrotoxin (Pribilla *et al.*, 1992; Zhang *et al.*, 1994, 1995; Enz and Bormann, 1995; Gurley *et al.*, 1995; Xu *et al.*, 1995), the leucine at position 301 is presumed to reside on the α-helical M2 domain and line the ion pore (Xu and Akabas, 1996).

Speculations on the mechanism by which the mutations affect activation. Our observations indicate that mutations of the leucine residue at position 301 may have extensive effects on gating of the homomeric ρ1 receptor that are difficult to reconcile with simple kinetic models. In particular, and as elaborated on below, it seems unlikely that a simple change from a normal desensitized state into a conducting state could account for the data (Revah *et al.*, 1991). Any interpretation must be speculative at this point, but some arguments can be made based on a qualitative survey of our results.

First, we consider the rapid reduction in current produced by low concentrations of GABA in the A, G, S, and T mutated subunits. This reduction could result from simple occlusion of the channel or by a mechanism involving more complex changes in kinetics. Simple occlusion is ruled out by several observations. First, the Hill coefficient for the antagonism by GABA is >1. A Hill coefficient of >1 would not be expected by a simple pore-blocking mechanism. Second, at higher GABA concentrations, the rapid reduction in current is replaced by a slow increase in current. This also is inconsistent with a continuing block. And, finally, the competitive antagonist 3-APMPA competitively antagonizes the reduction in current that also would not be expected for a pore-blocking mechanism.

Alternatively, the mutations may alter the gating kinetics of the receptor. Consider the wild-type activation mechanism in which three GABA molecules bind to fully activate the receptor (Amin and Weiss, 1996):

$$R \rightleftharpoons AR \rightleftharpoons A_2R \rightleftharpoons A_3R \rightleftharpoons A_3R^*$$

where R is the receptor, A is the agonist molecule, A_nR is the closed receptor bound with n agonist molecules, and A_3R^* is the open receptor with three bound agonist molecules. The

kinetic scheme can be modified to account for the spontaneously opening mutant receptors as follows:

$$\begin{array}{l} R \rightleftharpoons AR \rightleftharpoons A_2R \rightleftharpoons A_3R \rightleftharpoons A_3R^* \\ \Uparrow\Downarrow \\ R^* \end{array}$$

where R* is the spontaneous open state. In the absence of GABA, the receptors would be in equilibrium between the R and R* states. In accounting for the antagonism at low GABA concentrations, the binding of GABA would drive the receptors from the R state into the singly bound (AR) and doubly bound (A_2R) closed states. In this scenario, the binding of a single agonist molecule would be sufficient to stabilize the receptor in the closed conformation. The observation of a GABA-mediated channel closure seems in contradiction to a mechanism in which the binding of agonist stabilizes the subunit in an activated conformation, and when the requisite number of subunits are in this activated conformation, the receptor opens. This type of mechanism, in its simplest form, would not predict channel closing with agonist binding.

After the rapid reduction in current, GABA (at higher concentrations) produces a slow conductance increase when applied to receptors containing the A, G, S, and T mutant subunits. The fact that 3-APMPA on its own produces a slow increase in current with no rapid decrease would require that the processes of antagonism (discussed above) and activation produced by GABA would have to be uncoupled. The GABA-mediated activation observed at higher GABA concentrations could represent the normal pathway for channel activation with a decreased apparent sensitivity resulting from a decreased stability of the open state and/or a reduced cooperativity in agonist-mediated activation. A decrease in cooperativity is supported by the F, I, V, and Y mutations that demonstrate a dramatically reduced Hill coefficient for GABA-mediated activation (Table 3). Whatever the mechanism, normal gating must be altered since the resulting conductance increase resulting from GABA binding is both slower than normal and can be mimicked by 3-APMPA.

In summary, these observations are difficult to accommodate to a scheme that involves a unitary change, such as rendering a desensitized state conducting. Furthermore, the desensitized state should be nonresponsive to GABA. In fact, our spontaneously opening receptors do respond to GABA (i.e., they close). Instead, our data suggest that the energy landscape of receptor activation is altered in several respects by these mutations and a different mode of gating is revealed in the subunits with altered residues. Furthermore, they suggest that the kinetic states accessed in this mode do not map in a one-to-one fashion to states that can be discerned in the gating of the wild-type receptor.

Speculations on the role of this leucine in channel activation. The absolute conservation of the leucine at this position in the putative pore of all members of this receptor-operated family has focused considerable attention on its potential role in channel gating. Evidence suggests that the M2 domains of each of the five subunits line the pore (Leonard *et al*., 1988; Revah *et al*., 1991; White and Cohen, 1992; Karlin and Akabas, 1995; Xu and Akabas, 1996). In addition, evidence suggests these M2 domains are kinked helices and the invariant leucine located at the bend of all five subunits, through hydrophobic interactions with one another, prevents ion flux through the pore (Unwin, 1995). Destabilization of this leucine ring, prompted by agonist binding, weakens these interactions, allowing the leucine side-chains to rotate away from the central axis, thus opening the pore. This is only one model, and there is evidence using cysteine scanning mutagenesis that indicates cysteine residues substituted at positions intracellular to this putative gate-forming leucine are accessible in the absence of agonists. These studies place the gate more cytoplasmic than this conserved leucine (Xu and Akabas, 1996).

Muscle and neuronal nACh receptors, 5-HT_3 receptors, and heteromeric $\alpha1\beta2\gamma2$ GABA receptors still open (albeit with an increased agonist sensitivity) when the leucine is substituted with small polar residues. As discussed by Karlin and Akabas (1995), if this leucine formed the gate, it seems unlikely that it would still be operable with such nonconservative substitutions. In fact, these mutations in the homomeric $\rho1$ receptor (as well as the $\alpha1\beta1$ and $\alpha1\beta2\gamma2$ GABA receptor; Tierney *et al*., 1996) do prevent the normal agonist-dependent gating of the pore. Consistent with the hydrophobic interactions involving the five leucines maintaining the channel in the closed conformation, substitution of a hydrophilic residue (serine or threonine) or a hydrophobic residue with a smaller side chain (alanine and glycine) destabilized the closed state of the pore and allowed the receptors to open spontaneously (Chang *et al*., 1996).

Finally, one substitution merits discussion on its own (L301I). This substitution increased the hydrophobicity (Eisenberg *et al*., 1982) without altering the size of the side chain. Our prediction, based on the leucine-ring hydrophobicity hypothesis (Unwin, 1995), would be that substitution with isoleucine should induce a rightward shift in the dose-response relationship because greater energy (contributed by agonist binding) would be required to disrupt these hydrophobic interactions and open the pore. In fact, the EC_{50} value of the L301I mutant was indistinguishable from that of the wild-type receptor, although the rate of channel closing on agonist removal (deactivation) was decreased dramatically (11-fold). One possible interpretation of this result is that the leucine, perhaps through hydrophobic interactions with neighboring domains, also is important for maintaining the pore in the open state. Substitution with isoleucine, due to its slightly greater hydrophobicity (Eisenberg *et al*., 1982), further stabilizes the open state of the pore and impairs channel closure.

In conclusion, although this study is unable to assign the gate to L301, our results suggest this highly conserved leucine may play an important role in GABA-mediated activation of homomeric $\rho1$ GABA receptors. Determination of the precise role of this leucine residue in ligand-mediated activation ultimately will depend on the high-resolution structural imaging of the GABA receptor.

Acknowledgments

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