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Homomeric β1 γ-Aminobutyric acid$_A$ Receptor-Ion Channels: Evaluation of Pharmacological and Physiological Properties

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SUMMARY

The ubiquitous distribution of γ-aminobutyric acid$_A$ (GABA$_A$) receptor β subunits throughout the central nervous system is in accord with a vital role in receptor structure and function. Homomeric β subunits have been reported to be either GABA-gated or capable of forming anion-selective channels that lacked GABA-gating properties. With electrophysiological recording techniques, we examined the properties of the murine β1 subunit, addressed whether the homomeric receptor is expressed independently from the host cell's genome, and investigated whether these channels can open spontaneously. Murine β1 subunits, expressed in *Xenopus* oocytes or A293 cells, were unaffected by GABA or bicuculline; however, the resting membrane conductances were reduced by picrotoxin, zinc, or penicillin-G. In comparison, the expression of bovine β1 subunits formed GABA-gated Cl^- channels. For murine β1 subunits, both pentobarbitone and propofol increased the membrane conductance, although the benzodiazepine ligands flurazepam, flumazenil, and methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate were inactive. Oocytes injected with murine β1 cRNA in the presence of actinomycin D (to block host cell DNA transcription) expressed β1 channels that were indistinguishable from those derived from previous cDNA injections in cells capable of normal transcription. Single-channel recording from murine β1 cDNA-injected oocytes revealed spontaneously opening channels with a main state conductance of 18 pS. Picrotoxin inhibited the channel openings by reducing the probability of opening. We concluded that murine β1 subunits can form functional ion channels that are not gated by GABA but can be closed by some noncompetitive GABA antagonists. Interestingly, previous observations of spontaneously opening ion channels with properties similar to those found for the murine β1 receptor suggest that a limited expression of homomeric β subunit-ion channels may exist *in vivo*.

Molecular cloning studies of GABA$_A$ receptors have revealed a number of subunits that can be divided, based on a comparison of the deduced amino acid sequences, into the following four families and associated members: α1–6, β1–4, γ1–4, and δ1. Additional diversity in the structure of GABA$_A$ receptors is achieved by alternative splicing of the γ2, β2, and β4 subunit mRNAs (for review, see Refs. 1 and 2). Although it is believed that each GABA$_A$ receptor is composed of a pentameric arrangement of subunits (3), the precise subunit composition and stoichiometry of native GABA$_A$ receptors remain unknown.

It has become apparent, from expression studies, that the β subunit is a key structural and functional component of the GABA$_A$ receptor. Although a number of studies have reported expression of α or γ homomers or αγ heteromers (4, 5), robust receptor expression appears to rely on the presence of the β subunit, since coexpression of α and γ subunits often fails to produce functional GABA$_A$ receptors (4, 6, 7). From early biochemical studies, the β subunit was recognized as a potential binding site for GABA (8, 9). Moreover, a later investigation on recombinant GABA$_A$ receptors revealed that four amino acid residues near the amino terminus of the rat β2 subunit could form part of a GABA recognition site on the receptor (10).

Interestingly, expression of bovine or human homomeric β1 subunits has been shown to produce GABA-sensitive Cl^- channels in *Xenopus laevis* oocytes or human embryonic kidney cells (A293) (11–13). These GABA$_A$ receptors appeared to retain some allosteric binding sites, with pentobarbitone potentiating and PTX inhibiting the GABA-induced responses. Conversely, with a different species, the expression of rat β1 subunit homomers produced an anion-selective channel that lacked a sensitivity to GABA (14). These channels appeared to be open in the absence of GABA and could be closed by PTX (14), suggesting some degree of spontaneous channel opening in the absence of agonist. However, it is

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ABBREVIATIONS: GABA$_A$, γ-aminobutyric acid$_A$; MBM, modified Barth's medium; DMCM, methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate; A-9-C, antracene-9-carboxylic acid; ACh, acetylcholine; PTX, picrotoxin, Pen-G, penicillin-G; I-V, current-voltage; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid.

apparent that the spontaneous gating of these ion channels may be due to an artifact of, or interference from, the expression system (4).

To investigate the properties of the β subunit, we used electrophysiological methodologies to examine homomeric and heteromeric $GABA_A$ receptors expressed in *Xenopus* oocytes and transiently transfected A293 cells. In particular, we investigated novel pharmacological and physiological properties of murine β1 homomeric $GABA_A$ receptors and, for the first time, whether these ion channels can spontaneously open in the absence of agonist without the receptors being modified by any endogenous contribution from the host expression system.

Materials and Methods

Expression vectors. Murine β1 subunit[1] cDNA encoded for a polypeptide with amino acid sequence virtually identical (97%) to the equivalent rat β1 $GABA_A$ receptor polypeptide (15). The murine β1 cDNA was cloned as *Eco*RI fragments into the mammalian expression vector pGW1 as described previously (7).

Cell preparation: *Xenopus* oocytes. Oocytes were removed from anesthetized *Xenopus* as described previously (16) and placed into MBM consisting of 110 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 7.5 mM Tris·HCl, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, and 50 μg/ml gentamycin, pH 7.6. Oocytes at stages IV and V were centrifuged (700–1100 × *g* for 10 min at 10°) to reveal the nucleus, after which 15–20 nl of 1 mg/ml DNA solution, encoding for the murine or bovine β1 $GABA_A$ subunits, was injected into the nucleus. Injected oocytes were incubated at 19° for 2–5 days and fed every 2–3 days with fresh MBM. In some experiments, 50 nl of 1 mg/ml cRNA solutions encoding for the murine β1 subunit were injected into the oocyte cytoplasm. Oocytes were subsequently stored at 10° in MBM.

For two-electrode voltage clamp, the oocytes retained their follicular envelope. For single-channel recordings, the oocytes were manually defolliculated and devitelinized with the use of a hypertonic stripping solution (16, 17).

Human embryonic kidney cells. Human embryonic kidney cells (American Type Culture Collection CRL1573) were grown at 37° in 95% air/5% CO_2 in Dulbecco's modified Eagle's medium and Ham's F12 supplemented with 10% fetal calf serum (7, 18). Cells that were growing exponentially were seeded onto 35-mm plastic dishes for transfection with murine β1 cDNA, using a modified calcium phosphate technique (18). A293 cells were used for electrophysiological recording 18–36 hr after transfection and had membrane potentials of −30 to −40 mV.

Electrophysiology: Intracellular recording. Membrane currents and conductances were recorded from *Xenopus* oocytes using a two-electrode voltage-clamp technique. Oocytes were superfused with an amphibian Ringer's solution containing 110 mM NaCl, 2 mM KCl, 5 mM HEPES, and 1.8 mM $CaCl_2$, pH 7.4, at 8–10 ml/min (bath volume, 0.5 ml). Voltage and current microelectrodes were filled with 3 M KCl (5–10 MΩ) and 0.6 M K_2SO_4 (1–2 MΩ), respectively. Currents were recorded with an Axoclamp 2A amplifier in conjunction with a Gould Thermal Pen recorder.

Whole-cell and single-channel recording. Patch electrodes (1–5 MΩ) were filled with the following solution containing 140 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM HEPES, 11 mM EGTA, and 2 mM ATP, pH 7.1. The cells were continuously superfused in the culture dish with a Krebs' solution containing 140 mM NaCl, 4.7 mM KCl, 1.2 mM $MgCl_2$, 2.5 mM $CaCl_2$, 10 mM HEPES, and 11 mM glucose, pH 7.4. Membrane currents were recorded using an EPC7 patch amplifier. Series resistance compensation of 80% was routinely achieved, and membrane currents were filtered at 10 kHz (−3 dB, six-pole Bessel filter, 36 dB/octave).

For *Xenopus* oocytes, single-channel currents were recorded in cell-attached or outside-out patch configurations with the use of thin-walled electrodes coated with Sylgard (5–10 MΩ) and filled with either normal Krebs' (cell-attached patches) or with the following solution: 120 mM CsCl, 33 mM TEA-OH, 1 mM $MgCl_2$, 11 mM EGTA, 1 mM $CaCl_2$, 2 mM ATP, and 10 mM HEPES, pH 7.1 (outside-out patches). Single-channel data were stored on FM tape (DC to 5 kHz; Racal Store 4WD) and filtered at 1–3 kHz (−3 dB, six-pole Bessel). Records were digitized at 5–15 kHz and analyzed on a 486 PC using PAT version 6.6 software provided by Dr. John Dempster (University of Strathclyde, Glasgow, Scotland) as described previously (19).

Analysis of ligand-modulated membrane conductances. The ligand-induced membrane conductance change (ΔG) was calculated by subtraction from the resting conductance. Conductances were determined by the application of brief hyperpolarizing voltage command steps (1-sec duration, −10-mV amplitude, 0.2-Hz frequency) that were superimposed on the holding potential (−30 to −50 mV) in the absence and presence of a drug as described previously (7). These data were used to construct equilibrium concentration-response relationships for GABA and pentobarbitone (7). To pool dose-conductance data from more than one oocyte, all of the conductances were normalized (ΔG_N) to the conductance change produced by 10 μM of the appropriate ligand. The reductions in the resting membrane conductance by some $GABA_A$ receptor antagonists were used to construct antagonist concentration-inhibition relationships. These were obtained by defining the resting membrane conductance as 100% before the addition of the antagonist, and the data were fitted with an antagonist-inhibition model of the following form: $\Delta G_N'/\Delta G_N = [1 - [B/B + IC_{50}]]$, where $\Delta G_N'$ and ΔG_N represent the normalized agonist-induced conductance at a given concentration in the presence and absence of antagonist, respectively; *B* represents the antagonist concentration; and IC_{50} defines the concentration of antagonist producing a 50% inhibition of the agonist response.

I-V relationships. I-V relationships for the responses induced by the ligands were determined under voltage clamp. The holding potential was stepped with the use of depolarizing and hyperpolarizing voltage commands (10-mV increments, 1-sec duration) in the presence and absence of the ligand. In each case, the steady-state voltage step achieved and the induced current were measured. Finally, the ligand I-V relationship was determined by subtracting the resting leak I-V from the I-V relationship determined in the presence of the ligand.

Drugs and solutions. Diazepam, DMCM, flumazenil, and A-9-C were dissolved in the minimum quantity of 100% ethanol and pregnanolone was dissolved in acetone before use. Dilutions for application to oocytes were made with Ringer's solution, and the final concentration of ethanol or acetone did not exceed 0.03% (v/v). At this concentration of ethanol or acetone, all control recordings of drug-activated responses were unaffected by the solvents.

Results

Murine homomeric β1 subunits expressed in *Xenopus* oocytes: GABA-independent gating of the receptor-ion channel. At a holding potential of −40 mV, oocytes previously injected with β1 subunit cDNA were unaffected by GABA (0.1 μM–1 mM; Fig. 1A). However, the β1 cDNA-injected oocytes possessed input resistances that were significantly ($p < 0.05$) lower (0.2 ± 0.05 MΩ, mean ± standard error; 16 oocytes) than the input resistances measured on either uninjected oocytes or oocytes expressing murine α1β1 $GABA_A$ receptors (2.0 ± 0.5 MΩ; 22 oocytes). There were no visible morphological differences between the batches of oocytes, indicating that structural damage induced by the expression of homomeric β1 subunit receptors was unlikely.

[1] The term β1 subunit has been used to denote murine β1 subunits unless stated otherwise.

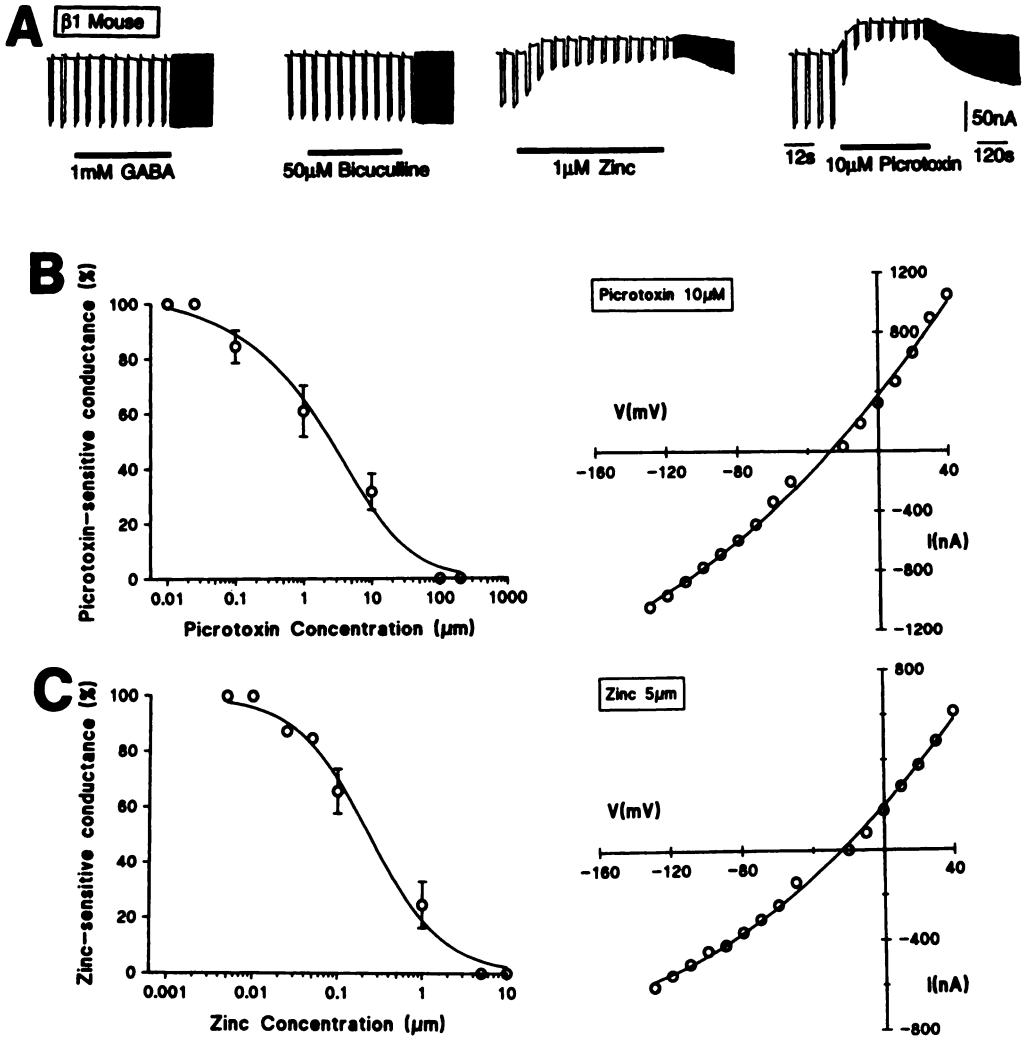


Fig. 1. Sensitivity to GABA and some antagonists of murine β1 homomeric $GABA_A$ receptors expressed in *Xenopus* oocytes. A, Bath application of 1 mM GABA, 50 μM bicuculline, 1 μM zinc, or 10 μM PTX. *Solid line*, duration of drug application; the membrane conductance was monitored throughout by repetitive application of brief hyperpolarizing voltage steps (−10 mV, 1 sec, 0.2 Hz). Holding potential, −40 mV. Note the two time calibrations; the chart recorder speed has been slowed 10-fold during the drug recoveries. The downward and upward deflections of each conductance step have been enhanced for clarity. B and C, Inhibition curves were constructed for the PTX- and zinc-sensitive membrane conductance (*left*). The control membrane conductance was defined as 100% before the addition of the antagonist. Data were fitted according to an antagonist-inhibition model (see Materials and Methods). Steady-state I-V relationships for the PTX- or zinc-sensitive currents were fitted using second-order polynomials (*right*). The reversal potentials for the PTX- and zinc-sensitive currents were −27.1 and −24.7 mV, respectively.

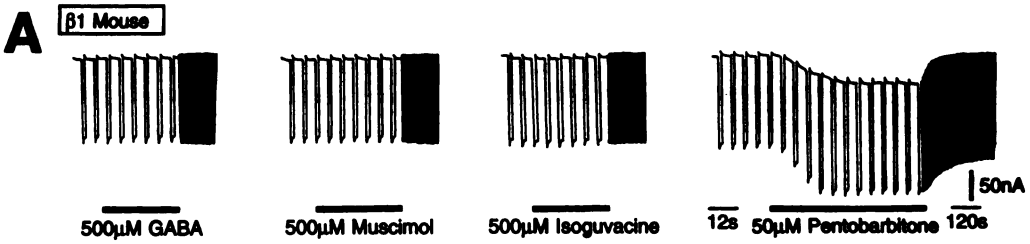
The low input resistance of β1 subunit-expressing cells suggested that some ion channels may be opening spontaneously in the membrane. Application of the competitive $GABA_A$ receptor antagonist bicuculline (50 μM) did not affect the resting membrane current or conductance (Fig. 1A), but low concentrations of either zinc (1 μM) or PTX (10 μM) restored the membrane resistance to control levels in these oocytes. Both antagonists produced reversible outward currents and conductance decreases in a concentration-dependent manner (Fig. 1A). PTX and zinc inhibition curves were constructed for the reductions in the membrane conductance (Fig. 1, B and C), yielding IC_{50} values of 2.11 ± 0.6 and 0.23 ± 0.03 μM (eight oocytes), respectively. PTX and zinc did not reduce the membrane conductance or induce outward currents in uninjected oocytes or in oocytes injected with α1β1 cDNAs.

I-V relationships in the presence and absence of these antagonists were used to assess the identity of the underlying conductance expressed after β1 cDNA injection. Both PTX and zinc appeared to target the same membrane conductance, with the I-V relationships displaying outward rectification with reversal potentials of −27 mV (PTX) and −24 mV (zinc). These values are close to the Cl^- equilibrium potential in these cells (Fig. 1, B and C).

GABA and pentobarbitone have separate binding sites on the murine β1 subunit. In addition to the lack of GABA-gating, the β1 homomeric channels were unaffected by muscimol (500 μM) and isoguvacine (500 μM; Fig. 2A). In contrast, pentobarbitone (50 μM) induced an inward current associated with a conductance increase (Fig. 2A). These results indicated that the binding sites for GABA and barbiturates are clearly different, with only the barbiturate site functionally existing in these homomeric β1 subunits. The equilibrium concentration-response curve for pentobarbitone produced an EC_{50} and Hill coefficient of 6.0 ± 0.34 μM and 1.9 ± 0.26, respectively (Fig. 2B). The I-V relation for the pentobarbitone-activated response revealed a reversal potential of −15.5 mV, which is similar to the Cl^- equilibrium potential in this batch of oocytes (Fig. 2B).

Modulation of murine β1 subunit gating by drugs acting at allosteric binding sites. Heteromeric $GABA_A$ receptors contain a number of allosteric binding sites (1, 2). The membrane Cl^- current gated by homomeric β1 subunits was unaffected by the benzodiazepine agonists flurazepam (10 μM) and midazolam (10 μM) or by the inverse agonist DMCM (10 μM; Fig. 3A). Moreover, the benzodiazepine antagonist flumazenil (10 μM; Fig. 3A), the neurosteroid pregnanolone (500 nM), and the nonsedative barbiturate chlormethiazole (100 μM) (20, 21) had no effect on this Cl^- current (Fig. 3B).

In contrast, propofol (100 μM), a novel general anesthetic



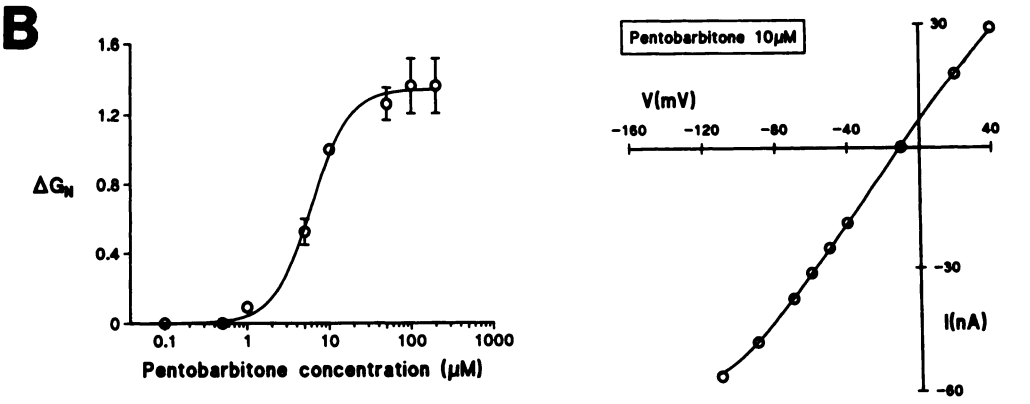
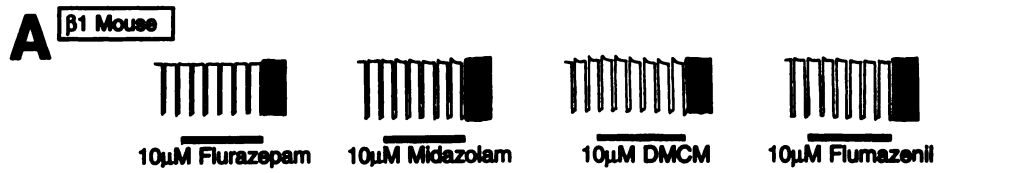
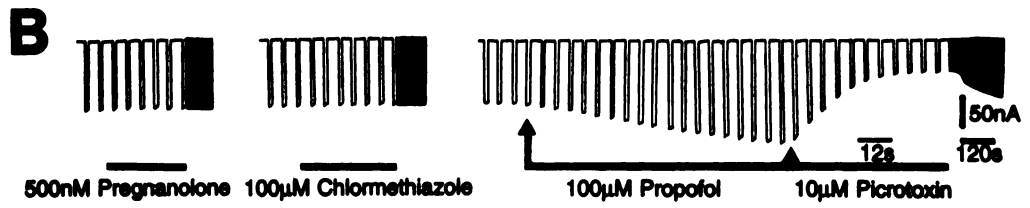


Fig. 2. Activity of GABA agonists and pentobarbitone on murine β1 homomeric receptors. *Solid line*, duration of drug application; the membrane conductance was monitored throughout by repetitive application of brief hyperpolarizing voltage steps (−10 mV, 1 sec, 0.2 Hz). A, Bath application of 500 μM GABA, 500 μM muscimol, 500 μM isoguvacine, or 50 μM pentobarbitone. B, Equilibrium concentration-response curve for the pentobarbitone-induced conductance (subtracted from the control membrane conductance), normalized with respect to the response evoked by 10 μM pentobarbitone (*left*). The points represent mean ± standard error recorded from five oocytes. The curve was fitted according to the logistic model (7). The I-V relationship for the response to 10 μM pentobarbitone yielded a reversal potential of −15.5 mV (*right*).





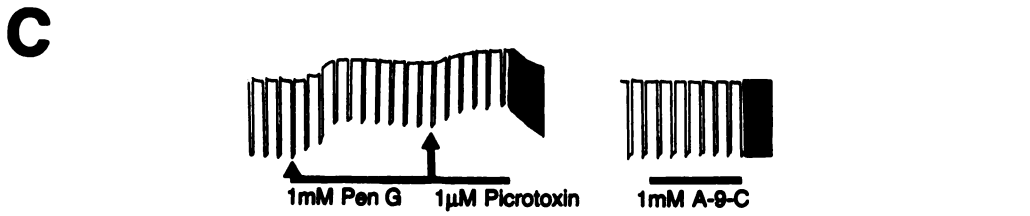


Fig. 3. Effect of $GABA_A$ receptor allosteric modulators on the homomeric murine β1 receptor expressed in *Xenopus* oocytes. *Solid line*, duration of drug application; the membrane conductance was monitored throughout by repetitive application of brief hyperpolarizing voltage steps (−10 mV, 1 sec, 0.2 Hz). A, Application of 10 μM flurazepam, 10 μM midazolam, 10 μM DMCM, or 10 μM flumazenil. B, Application of 500 nM pregnanolone, 100 μM chlormethiazole, and 100 μM propofol in the absence and presence of 10 μM PTX. C, The effect of 1 mM Pen-G, either alone or in combination with 1 μM PTX, on the membrane current and conductance. *Solid line*, 1 mM A-9-C application.

and positive modulator of GABA-activated responses (22), increased the membrane conductance that could be inhibited by PTX (10 μM; Fig. 3B). This action of propofol presumably involved enhancing the current through the β1 homomeric ion channels, since the resting membrane resistances of uninjected oocytes were unaffected by this agent.

Pen-G is an inhibitor of GABA-activated currents and may act as an open Cl^- channel blocker (23, 24). Application of 1 mM Pen-G induced an outward current and conductance decrease that could be accentuated by the coapplication of PTX (1 μM; Fig. 3C). Pen-G had no effect on the resting membrane resistance of murine α1β1 cDNA-injected or noninjected oocytes. Thus, Pen-G and PTX appear to be inhibiting the same ion current formed by the homomeric β1 subunit. In contrast, an inhibitor of voltage-gated Cl^- channels, A-9-C (1 mM) (25), had no effect on the membrane current or conductance (Fig. 3C).

Murine β1 subunit ion channel: A possible artifact of endogenous expression from the oocyte's genome? *Xenopus* oocytes have been extensively used as expression systems to study post-translationally processed membrane-bound ion channels (26). However, it is conceivable that

under some circumstances, transcription of the oocyte's genome may interfere with the expression of foreign protein. For the β1 $GABA_A$ receptor subunit, coassembly with a transcription product from the oocyte's genome may affect gating properties, leading to apparently spontaneous channel opening.

To control for oocyte genomic transcription, control experiments were performed to determine whether the Cl^- current observed in this study was a product solely of the β1 subunit expression. The first approach was to inject into the *Xenopus* oocyte the *Lac Z* gene, which encodes for the β-galactosidase protein (27). This sham-injection was used to test whether the persistently activated Cl^- current could be induced by initiating transcription of a foreign gene in the oocyte's nucleus. After the injection, *Lac Z* cDNA-injected oocytes exhibited resting membrane resistances similar to those of control uninjected oocytes. Furthermore, GABA (1 mM), zinc (10 μM), PTX (50 μM), or pentobarbitone (50 μM) had no effect on the resting membrane current or conductance (Fig. 4A). It is therefore improbable that the injection of cDNAs *per se* activates an endogenous gene within the oocyte that could be expressing a channel protein that is sensitive to some $GABA_A$ receptor modulators and responsible for the Cl^- current.

The second approach, to differentiate β1 subunit expression from the host cell's genomic transcription, involved incubating oocytes in the presence of 50 μg/ml actinomycin D after injection with cRNA encoding for the β1 subunits. The effectiveness of actinomycin D was assessed within the same series of experiments by injecting cDNAs encoding for murine α1β1 $GABA_A$ receptors into the nucleus. As actinomycin D blocks transcription of DNA to mRNA, the translation of the β1 cRNAs should be unaffected by actinomycin D, but the expression of the α1β1 subunits after cDNA injection should be prevented. After 2–5 days' incubation in actinomycin D, the β1 cRNA injection resulted in functional β1 subunits possessing the same ion channel properties as those seen for cDNA injections of the β1 subunit. GABA (500 μM) failed to affect the ion channel properties, but both zinc (1 μM) and PTX (50 μM) induced outward currents and conductance decreases (Fig. 4B). Moreover, pentobarbitone (10 μM) induced an inward current and conductance increase (Fig. 4B). To ensure that actinomycin D was preventing transcription at this concentration, oocytes injected with α1β1 cDNAs were also bathed in actinomycin D for 2–5 days. Expression of α1β1 subunits was not observed, and both GABA (10 to 500 μM) and PTX (50 μM) failed to elicit any response (Fig. 4C). To determine whether this lack of expression was due to the effects of actinomycin D and not to poor expression of the cDNAs, oocytes from the same experiment were also incubated in the absence of actinomycin D. In this case, the injection of α1β1 subunits for the $GABA_A$ receptor led to the robust and efficient formation of GABA-gated anion channels. GABA (10 and 100 μM) induced large inward currents and conductance increases that were inhibited by PTX (10 μM; Fig. 4D).

Expression of bovine homomeric β1 subunits. To further validate the pharmacological profile of the murine β1

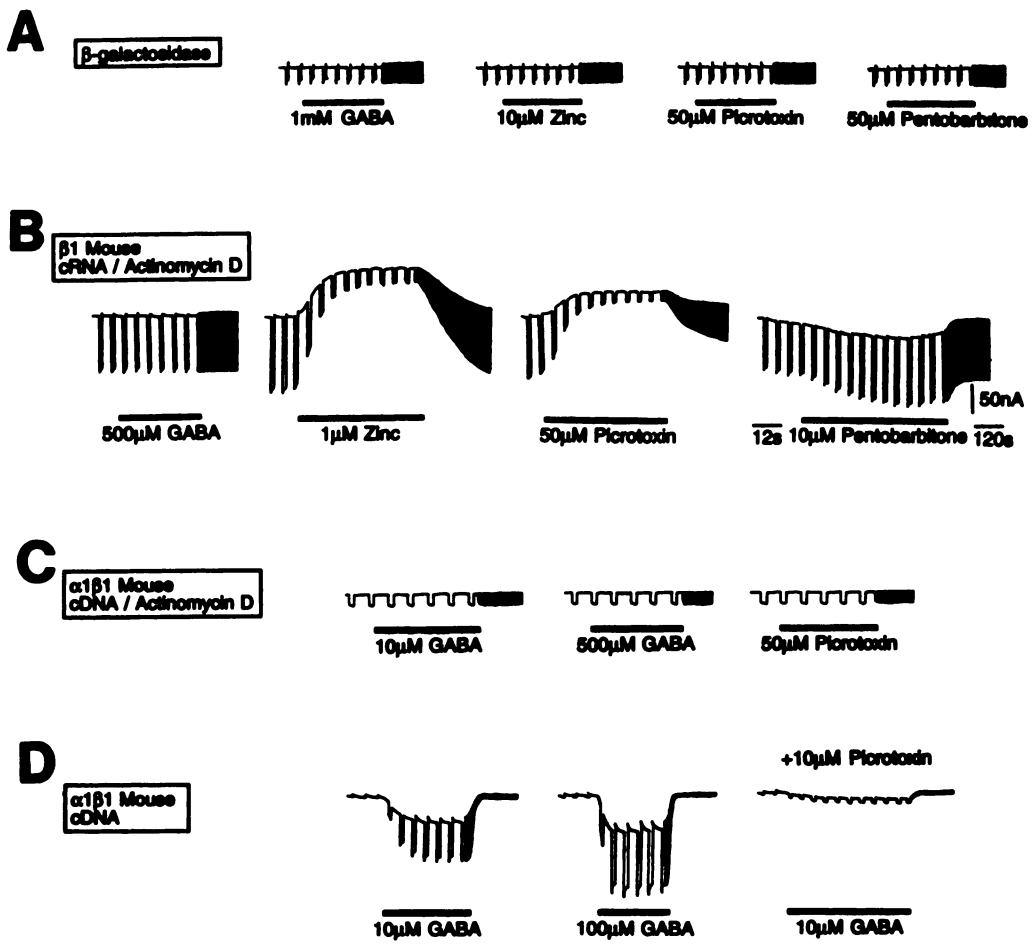


Fig. 4. Effect of endogenous genomic transcription on the expression of murine β1 subunits in *Xenopus* oocytes. *Solid line*, duration of drug application; the membrane conductance was monitored throughout by repetitive application of brief hyperpolarizing voltage steps (−10 mV, 1 sec, 0.2 Hz). A, Pharmacological sensitivity to 1 mM GABA, 10 μM zinc, 50 μM PTX, and 50 μM pentobarbitone of oocytes injected with the *Lac Z* gene encoding for β-galactosidase. B, Injection of cRNA corresponding to the mouse β1 subunit. Oocytes were incubated in 50 μg/ml actinomycin D for 2–5 days before assessing their sensitivity to 500 μM GABA, 1 μM zinc, 50 μM PTX, and 10 μM pentobarbitone. C, Injection of cDNAs corresponding to α1β1 $GABA_A$ subunits. After incubation, conditions described in B, 10 and 500 μM GABA or 50 μM PTX was bath-applied. D, Injection of cDNAs corresponding to α1β1 $GABA_A$ receptor subunits into oocytes in the absence of actinomycin D. Evoked responses are shown to 10 and 100 μM GABA and the antagonism of the response to 10 μM GABA by PTX (10 μM).

subunits, oocytes were injected with bovine β1 cDNA. These expressed subunits were clearly different than their murine counterparts, as both GABA (0.1 μM–1 mM) and muscimol (100 μM) evoked a distinct conductance increase (Fig. 5, A and C). Moreover, bovine β1 subunits were not spontaneously opening because PTX (10 μM) and zinc (10 μM) failed to induce any outward currents, but both of these antagonists and bicuculline (10 μM) inhibited the response evoked by 100 μM GABA (Fig. 5B). The benzodiazepine midazolam (≤50 μM) failed to enhance the GABA-activated response, but pentobarbitone (50 μM) induced a small enhancement (Fig. 5A). The concentration-response curve to GABA had an EC_{50} value of 29.34 ± 5.1 μM (Fig. 5D).

Expression of persistently activated homomeric murine β1 subunits is independent of the expression system. To determine whether the Cl^- current was a feature or artifact of expression in *Xenopus* oocytes (4), we also expressed murine β1 subunits in A293 cells. During whole-cell recording at a holding potential of −50 mV, the application of 20 μM GABA or 10 μM bicuculline from an adjacent multibarrelled pipette did not induce a change in the membrane current (Fig. 6, A and B). Furthermore, as for the murine β1 homomeric ion channel expressed in the oocytes, application of either zinc (100 μM) or PTX (10 μM) evoked outward currents with concurrent reductions in membrane conductance (Fig. 6, A and B). In control untransfected cells, PTX (10 μM) and zinc (100 μM) did not affect the resting membrane current or conductance (Fig. 6C).

Single-channel properties of murine α1β1 and β1 $GABA_A$ receptors. To isolate the ion channel events underlying the persistently activated, PTX-sensitive Cl^- current, single-channel currents were recorded from *Xenopus* oocytes injected with either murine β1 or α1β1 cDNAs. In outside-out patches, obtained from oocytes expressing α1β1 subunit-containing receptors, 5 μM GABA activated single-channel currents with amplitudes of 1.32 and 0.9 pA corresponding to a main conductance state of 16 pS and a far less frequently observed subconductance state of 11 pS at −80 mV patch holding potential.

The gating properties of the α1β1 channels were analyzed from the frequency distributions of all open and closed durations (Table 1). The open-time frequency histograms were routinely fitted by two exponential functions, requiring mean time constants of 0.41 ± 0.04 and 1.71 ± 0.08 msec (five patches). The closed-duration histograms were fitted by the sum of three exponential functions, designated as short, intermediate, and long (Table 1).

Xenopus oocytes expressing β1 subunits were studied in cell-attached and outside-out patch configurations and principally identified by their presence only in oocytes injected with β1 subunit cDNA and by their sensitivity to block by PTX. Single-channel currents were recorded in cell-attached patches with various amplitudes of 1.4, 2.5, and 3.7 pA at a patch potential of −100 mV (corresponding to transpatch potentials of −130 to −140 mV, as determined after rupture of the cell-attached patch to record the resting potential; resting potentials were also confirmed by measurement with intracellular recording electrodes). These channel currents corresponded to the following conductance states: a main conductance of 18 pS, with a less frequently occurring supraconductance state of 26 pS and a rare subconductance state of 10 pS (Fig. 7 and Table 1). The last state was too rare to feature prominently on the single-channel current amplitude histogram.

Individual openings demonstrated transitions from 18 to 26 pS and 26 to 18 pS states, in addition to 18 to 10 pS and returning to 18 pS transitions. The 10 pS state was not seen as a discrete opening from and returning to the base-line (Fig. 7). The gating properties of the channels revealed that they were frequently open but also able to enter into closed periods.

In outside-out or cell-attached patches, open-time distributions were described by two exponential components with time constants of 0.39 and 1.92 msec (Fig. 8A and Table 1). Unlike the closed-time histograms for α1β1 subunit receptors, the closed-time distributions for homomeric β1 channels were adequately described by only two exponential components with constants of 0.1 and 0.88 msec (Fig. 8B and Table 1). The identity of the channels was assessed by the reversal potential for the channel currents. Stepping the patch pipette potential from −80 mV to +20 mV resulted in a reversal potential close to 0 mV, the predicted Cl^- equilibrium rever-

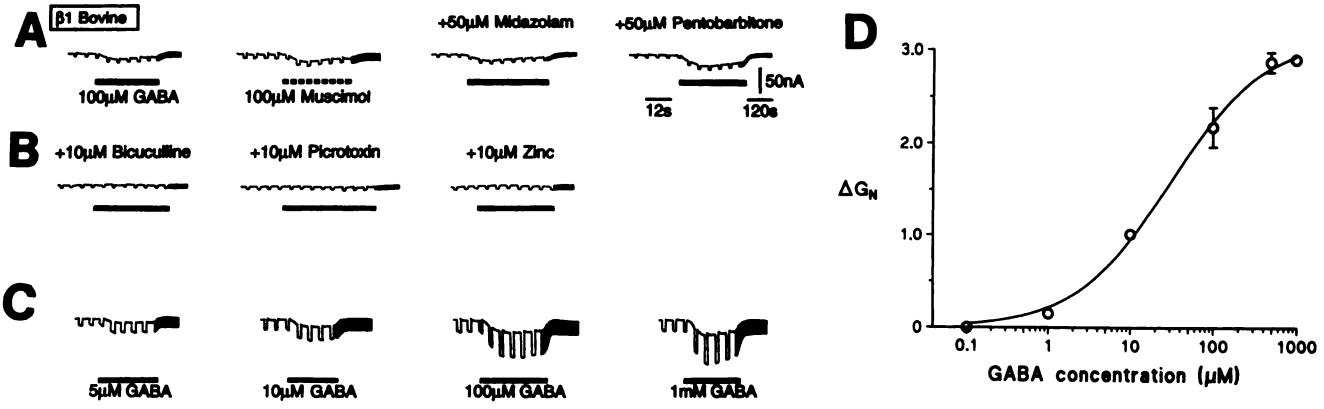


Fig. 5. Properties of bovine β1 subunits expressed in *Xenopus* oocytes. *Solid line*, duration of drug application; the membrane conductance was monitored throughout by repetitive application of brief hyperpolarizing voltage steps (−10 mV, 1 sec, 0.2 Hz). A, Cells were exposed to GABA (100 μM) and muscimol (100 μM) or GABA (100 μM) in the presence of midazolam (50 μM) or pentobarbitone (50 μM). B, Responses to 100 μM GABA in the presence of bicuculline (10 μM), PTX (10 μM), or zinc (10 μM). C, In a different oocyte, responses to 5 μM–1 mM GABA. D, Equilibrium concentration-response curve for the GABA-induced conductance normalized with respect to the response to 10 μM GABA (three oocytes). Data are mean ± standard error. The curve was fitted according to the logistic model (7).

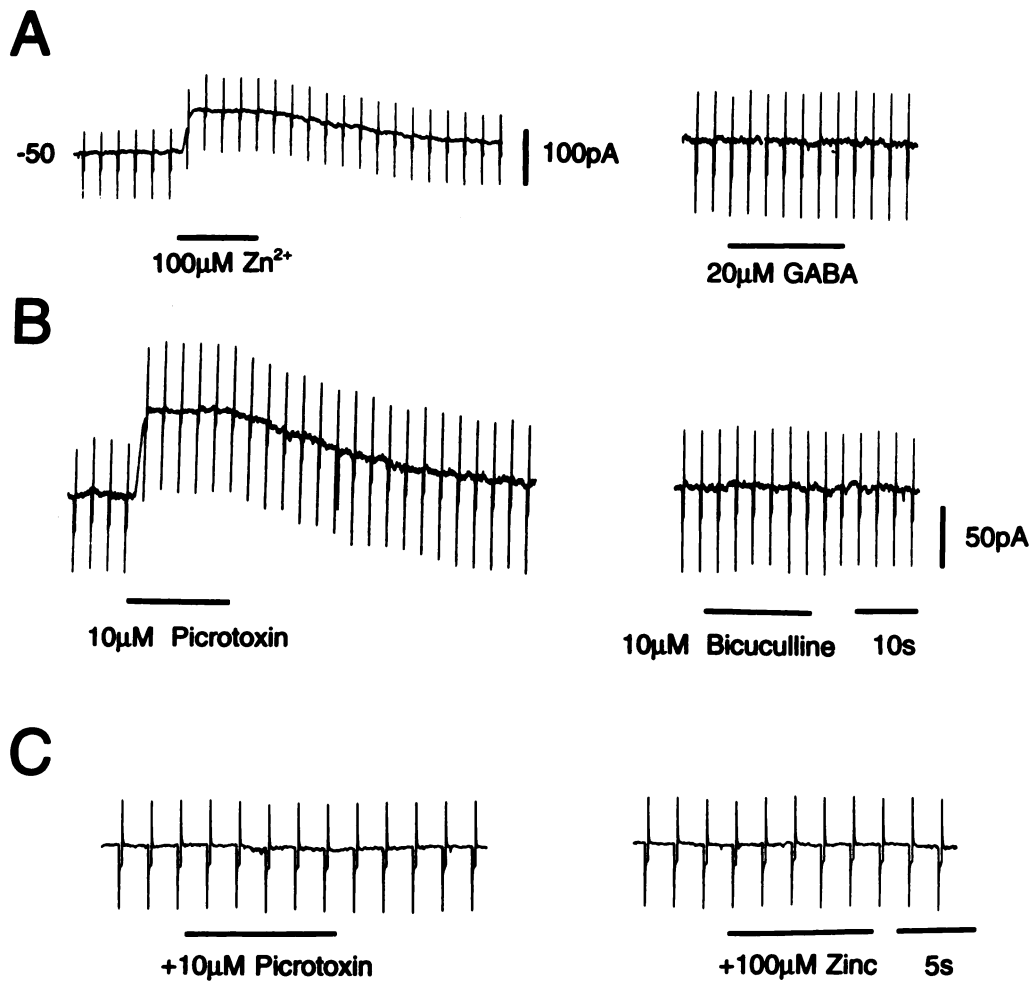


Fig. 6. Pharmacology of murine $\beta 1$ $GABA_A$ receptor subunits expressed in transfected A293 cells. *Solid line*, duration of drug application. Whole-cell membrane currents and conductances were monitored in transfected cells during exposure to (A) 100 μM zinc or 20 μM GABA; (B), 10 μM PTX or 10 μM bicuculline; and (C), in untransfected control cells, to 10 μM PTX or 100 μM zinc. Responses were derived from three different cells. Holding potential, −50 mV. Conductance was measured using hyperpolarizing voltage commands (−10 mV, 300 msec, 0.3 Hz).

TABLE 1

Single-channel properties of murine $\alpha 1\beta 1$ and $\beta 1$ recombinant $GABA_A$ receptors

Single-channel currents through $\alpha 1\beta 1$ ion channels were activated by 5 μM GABA either superfused over an outside-out patch or included in the patch pipette electrolyte for cell-attached patches. For $\beta 1$ cDNA-injected oocytes, ion channel currents occurred spontaneously in the absence of GABA. The values were accrued from three to five patches and represent mean ± standard error. Details of the analyses have been described previously (19).

$GABA_A$ receptor construct	$\alpha 1\beta 1$	$\beta 1$
Main conductance state (pS)	16	18
Subconductance states (pS)	11	10
Supraconductance states (pS)		26
Mean open times		
τ_{O1} (msec)	0.41 ± 0.04	0.39 ± 0.15
τ_{O2} (msec)	1.71 ± 0.08	1.92 ± 0.32
Mean closed times		
τ_{C1} (msec)	0.65 ± 0.02	0.1 ± 0.05
τ_{C2} (msec)	9.36 ± 2.12	0.88 ± 0.05
τ_{C3} (msec)	21.98 ± 1.98	
P_o	0.08 ± 0.03	0.03 ± 0.014

sal potential for these outside-out patches. Moreover, similar to the whole-cell persistently activated currents, these single channel currents were also inhibited by externally applied 10 μM PTX (Fig. 8E). This antagonist did not affect the conductance states (18 or 26 pS) of the channel, although the 10-pS state was now too infrequent to be monitored, but the probability of channel opening (P_o) was reduced [from 0.03 ± 0.014 (control) to 0.009 ± 0.005 (+PTX)]. Analysis of the channel gating revealed that PTX did not affect the open-time constants [τ_{O1} of 0.39 ± 0.15 msec and τ_{O2} of 1.92 ± 0.32 msec (controls) to 0.25 ± 0.13 and 1.67 ± 0.1 msec (+PTX)] but slightly reduced the relative frequency of longer openings [from 55% (control) to 42% (+PTX)] while increasing the relative frequency of short openings [from 45% (control) to 58% (+PTX; Fig. 8C)]. The closed-time distribution in the presence of PTX now required three time constants to describe the histogram (τ_{C1}, 0.51 ± 0.07 msec; τ_{C2}, 2.9 ± 1.1 msec; τ_{C3}, 68 ± 19 msec; Fig. 8D). A long time constant (τ_{C3}) was revealed that previously was not resolved in these frequently active channels, and both the short and intermediate closed time constants were increased compared with controls (Fig. 8, B and D, and Table 1). This effect of PTX was evident on single-channel currents measured in $\beta 1$ subunit cDNA-injected oocytes but not in uninjected oocytes.

Discussion

Pharmacological Profile of Murine Homomeric $\beta 1$ $GABA_A$ Receptors

Expression of murine $\beta 1$ subunits resulted in the formation of anion-selective channels that lacked the ability to be gated by GABA and were apparently capable of opening in the absence of an agonist. This was indicated by a low resting membrane resistance that was increased by the application of PTX or zinc. In accord with the lack of sensitivity to

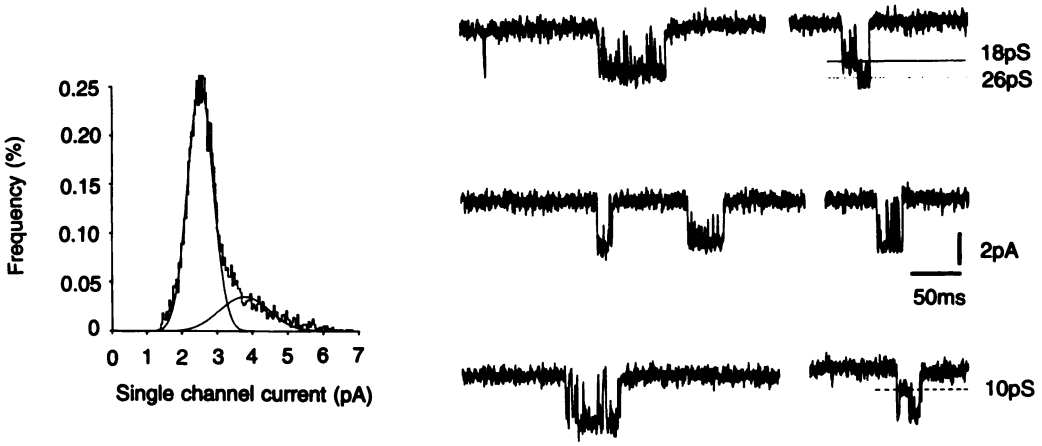


Fig. 7. PTX-sensitive single-channel currents in oocytes expressing murine β1 subunits. Representative examples of single-channel currents and associated transitions in the channel conductance in a cell-attached patch (patch potential of −100 mV, corresponding to a transpatch potential of −140 mV, after determination of the cell membrane potential). This patch demonstrated openings to three current levels at 2.5 and 3.7 pA and a rare state at 1.4 pA (corresponding to 18, 26, and 10 pS conductance states, respectively). Records are filtered at 3 kHz (see Materials and Methods). The single-channel current amplitude distribution was generated from a single oocyte. Two gaussian components were required to fit the data with mean value of 2.53 and 3.7 pA (note the 10 pS conductance state was sufficiently rare to not feature prominently on the histogram). Chord conductances were calculated to be 18.1 and 26.4 pS.

muscimol and isoguvacine, the β1 receptor-mediated current was also insensitive to the competitive $GABA_A$ receptor antagonist bicuculline, suggesting that the binding site or sites are either not present or functionally inactive.

Murine β1 Subunit Ion Channels Are Formed after cDNA Transcription

A previous study reported that *Xenopus* oocytes have the ability to express an ACh receptor α subunit after transcription of an endogenous gene. The resultant polypeptide was thought to coassemble with ACh receptor subunit proteins, synthesized *de novo* after cRNA injection, to yield functional ACh receptors (28). Furthermore, A293 cells have been shown to produce $GABA_A$ receptor β3 subunit mRNA (29). Therefore, it was important to ensure that the unusual ion channel formed from injection of cDNAs encoding for murine β1 subunits in *Xenopus* oocytes was not occurring due to expression of an endogenous gene product. This was determined by the use of a transcription blocker, actinomycin D. Injection of β1 cRNAs and subsequent expression of the translation products were unaffected by actinomycin D. Therefore, it is likely that the channel currents seen in this study arose only after the expression of homomeric murine β1 subunits and did not result from a combined product originating from the oocyte's genome. Moreover, because outward currents to PTX were also observed in A293 cells, this indicates that these β1 ion channels are not an artifact of the oocyte expression system.

Spontaneously Opening Ion Channels in Murine β1 cDNA-Injected Oocytes

Ion channel currents were measured from α1β1 subunit combinations to assess the translational competence of the batches of oocytes used in this study. The ion channel conductances, their sensitivity to GABA, and their gating kinetics were all in accordance with previous studies (6, 30, 31).

The spontaneous ion channel currents that may underlie the whole-cell currents resolved in *Xenopus* oocytes injected with murine β1 cDNAs were isolated in cell-attached and outside-out patches. Single-channel currents reversed at 0 mV, suggesting mediation by Cl^-, and were blocked by PTX. The gating properties of the channels were completely unaffected by the presence of GABA, but in contrast, PTX reduced the probability of opening and decreased the proportion of long channel openings while increasing the residence time in long closed durations. Some of these findings are similar to those reported for PTX-blocking recombinant murine α1β1 $GABA_A$ subunit receptors in Chinese hamster ovary cells (31) and native $GABA_A$ receptors in spinal neurons (32) or autonomic ganglia (33). This suggests that homomeric $GABA_A$ receptor channels may be structurally quite similar with regard to PTX block compared with their more complex counterparts formed from combinations of α, β, and γ subunits and channels formed from the currently unknown compositions of native neuronal $GABA_A$ receptors. Comparison of the conductance states also reveals further similarities: β1 channels form at least three states, 10, 18, and 26 pS, whereas α1β1 subunits formed channels with states of 10 and 16 pS. It is interesting that previous studies have reported 10 and 15 pS states for α1β1 and 21 and 29 pS states for α1β1γ2 (6, 30). The similar multiples of conductances again suggest some commonality between the ion channels formed from heteromeric and homomeric $GABA_A$ receptor ion channels.

Contamination of our single-channel records by stretch-activated channels or endogenous Ca^{2+}-activated Cl^- channels was unlikely. We used narrow pipette diameters to limit the activity of the stretch-activated channels, and they possess a different main conductance state of 28 pS (17) compared with the spontaneous channels observed in the present study. Activation of the voltage- and Ca^{2+}-dependent Cl^- channels was reduced by using negative holding potentials and controlling the pipette free Ca^{2+} concentration with EGTA in outside-out patches.

Comparison with Previous Results

β1 ion channels. An interpretation of previous studies suggests that the properties of expressed β1 subunits may depend on the species of cDNA used; however, β1 subunits from different species have not been compared within a single functional study. In our investigation, bovine homomeric

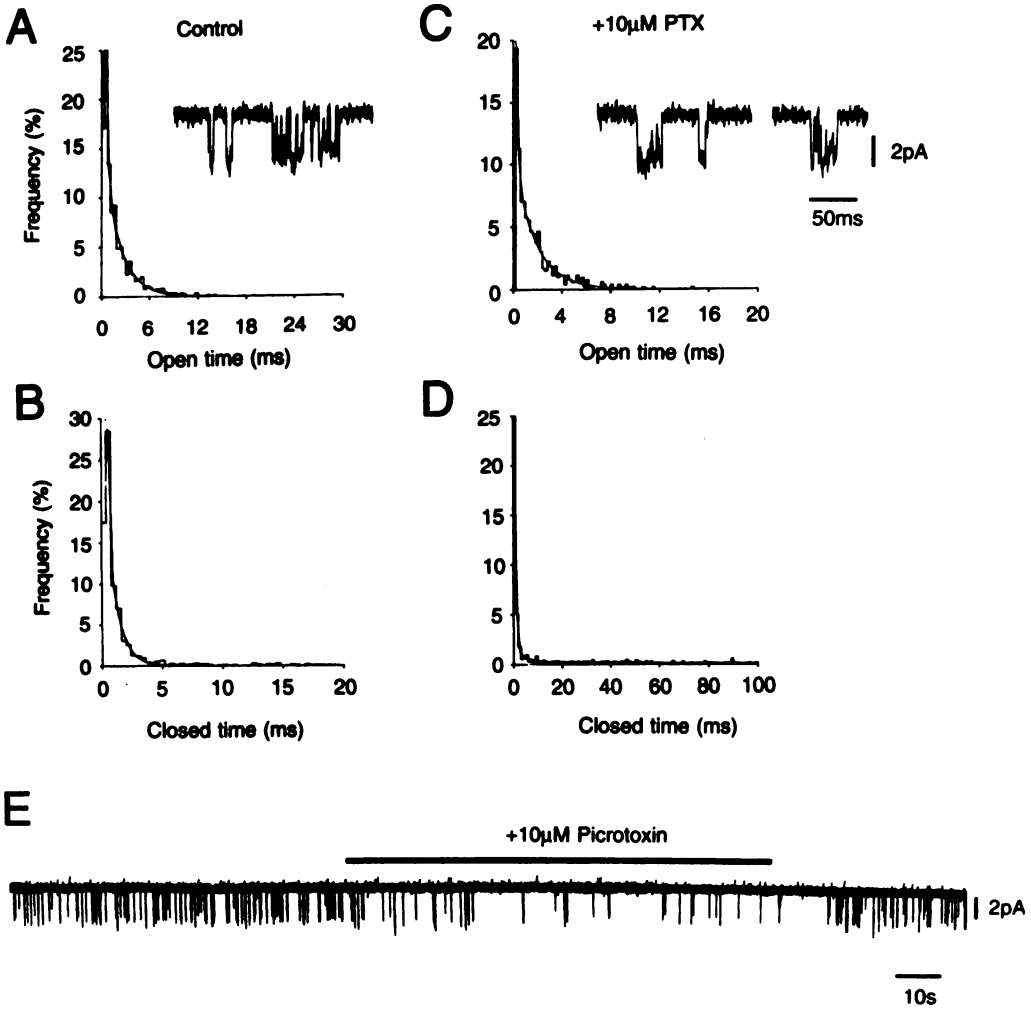


Fig. 8. Open and closed dwell time frequency histograms for spontaneously opening channels in outside-out patches taken from *Xenopus* oocytes injected with murine β1 cDNA. Data were obtained before (A and B) and after (C and D) exposure to 10 μM PTX. *Insets*, sample single-channel currents taken in the absence (A) and presence (C) of PTX. The patches were held at −140 mV. The mean open-time constants were 0.28 and 2.15 msec in control Krebs' and 0.15 and 1.73 msec in the presence of PTX. The closed-time distribution in control Krebs required two exponential components, with time constants of 0.14 and 0.9 msec, which changed in PTX to requiring three exponentials with time constants 0.56, 3.65, and 82.2 msec. E, Single spontaneous ion channel currents recorded in an outside-out patch taken from a murine β1 cDNA-injected *Xenopus* oocyte in the absence and presence of 10 μM PTX.

β1 $GABA_A$ receptors, when compared with murine β1 subunits, apparently did not open spontaneously (see Ref. 11). These data are compatible with species-dependent differences in the functional properties of β1 subunits. For bovine β1 receptors, the EC_{50} value for GABA of ~30 μM is 5-fold higher than the EC_{50} value of 5.87 ± 0.6 μM for the heteromeric α1β1 $GABA_A$ receptor construct (7), indicating a lower affinity and/or efficacy of GABA for the bovine β1 $GABA_A$ receptor. Furthermore, human β1 homomeric $GABA_A$ receptors expressed in A293 cells (12) and *Xenopus* oocytes (13) are also gated by GABA.

In comparison, rat β1 subunits mediated PTX-sensitive outward currents and lacked GABA-gating properties (14). Moreover, application of PTX to oocytes expressing rat "β" $GABA_A$ receptors also elicited an outward current (34). Rather surprisingly, PTX-induced currents were also observed in heteromeric $GABA_A$ receptors expressed in oocytes injected with either α1 or α5 and "β" RNAs (34). Similar currents were observed in oocytes expressing rat α1β1γ2 $GABA_A$ receptors (35). It is possible that PTX-sensitive currents mediated by heteromeric GABA receptors could be due to a minor proportion of channels open in the absence of added GABA or that these "open channels" could represent the expression of a small population of homomeric β1 $GABA_A$ receptors. Interestingly, the main differences between the amino acid sequences of murine and rat and those of bovine and human β1 subunits occur in the large putative intracellular loop between transmembrane domains M3 and M4. It is therefore conceivable that changes of amino acid residues in this intracellular region could incur conformational changes in the $GABA_A$ receptor, leading to the pharmacological profiles seen in this and previous studies. However, we cannot discount the possibility of differential post-translational processing also accounting for these differences in gating.

β1 subunit pharmacology. The current passing through the murine β1 ion channel was enhanced directly by pentobarbitone, providing a clear indication that GABA and barbiturates must bind to different sites within the $GABA_A$ receptor complex. In the present study, the Hill coefficient for pentobarbitone was 1.9 ± 0.26, which may be interpreted as indicating two binding sites for pentobarbitone on the homomeric murine β1 subunit. Pentobarbitone can enhance the GABA-induced response in human β1 homomeric $GABA_A$ receptors expressed in *Xenopus* oocytes (13).

Conversely, the benzodiazepines and the neurosteroid pregnanolone appear to have no binding sites on murine β1 $GABA_A$ receptors. These results are compatible with previous studies using heteromeric constructs, where the benzodiazepine enhancement of GABA-induced responses on $GABA_A$ receptors occurs only when a γ subunit was present. The selectivity for the benzodiazepines was determined by the type of α subunit (36). Similarly, neurosteroid modula-

tion of the $GABA_A$ receptor was also strongly influenced by the presence of both the α and the γ subunits but not by the β subunits (37). In contrast, alphaxalone potentiated the GABA-activated response in human β1 homomers in *Xenopus* oocytes (13). This difference could occur due to the different species of β1 expressed or possibly the different neurosteroid studied.

Interestingly, propofol potentiated the murine β1 ion channel conductance, demonstrating a potential binding site for this general anesthetic that is separate from the GABA and benzodiazepine binding sites but possibly shared with the barbiturate binding site. Propofol has been previously shown to act as a positive allosteric modulator at $GABA_A$ receptors, although no firm conclusions were drawn as to the site of action on the receptor (22). Recently, a direct action of propofol at human β1 homomeric $GABA_A$ receptors was observed with a small enhancement in the response to GABA (13).

The noncompetitive antagonists zinc, PTX, and Pen-G have the ability to antagonize the currents mediated by murine β1 channels, suggesting the unequivocal existence of binding sites that are discrete from the agonist recognition site on β1 subunits.

Can $GABA_A$ Receptor-Ion Channels Open Spontaneously?

This question has been posed many times for neurotransmitter-gated ion channels. Spontaneously opening $GABA_A$ receptor-ion channels are difficult to identify in tissue culture or *in situ* because in the absence of any exogenously applied agonist, a background release of endogenous GABA may cause channel activation. Identification of spontaneous currents has to rely on pharmacology and on an analysis of channel-gating properties. Single-channel events of similar conductance (24 pS) to GABA-activated ion channels have been reported in spinal neurons (38). Spontaneous Cl^--mediated single-channel currents have also been observed in pituitary cells (39, 40), where conductances of 11, 23 (main state), and 31 pS were reported. These conductances and the spontaneous channel-gating kinetics are similar to those of GABA-activated channels in these same cells, although GABA caused more prolonged bursts of channel activity (39). The spontaneous channels were unaffected by bicuculline or SR 42641, suggesting that low concentrations of an agonist (GABA is probably absent from these cells) are unlikely to be activating the channels (39). In contrast, $GABA_A$ receptor antagonists that may block the Cl^- channels, such as *t*-butylbicyclophosphorothionate and PTX, were capable of inhibiting the spontaneous channel activity (40). *t*-Butylbicyclophosphorothionate increased the long closed durations of the channel in a similar manner to that of PTX, inhibiting the spontaneous channels recorded in our murine β1 cDNA-injected oocytes. One interpretation of these data is that in some cells, spontaneously opening channels can occur with properties similar to those of GABA-activated channels. These channels may be *bona fide* $GABA_A$ receptors (e.g., composed of α, β, and γ subunits) or could represent some limited expression of homomeric β1 subunits. To establish the likelihood of homomeric receptor expression occurring in the presence of other $GABA_A$ receptor subunit expression will require some understanding of the rules governing $GABA_A$ receptor assembly.

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