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Effects of Chlorotrifluoroethylene Oligomer Fatty Acids on Recombinant GABA Receptors Expressed in *Xenopus* Oocytes

N.J. DelRaso, Y. Huang, L. Lu
Department of Physiology and Biophysics, Wright State University School of Medicine, Dayton, OH 45435

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Abstract. GABA-activated Cl^- current was expressed in *Xenopus* oocytes after injecting cRNA that had been transcribed in vitro from complementary DNA (cDNA) coding for a single GABA ρ_i-subunit cloned from human retina. The expressed current was insensitive to 100 μM bicuculline, but was activated by the GABA analogue trans-4-aminocrontonic acid (TACA). Anion-selective permeability of the expressed ρ_1-subunit was determined by isotonically replacing the extracellular Cl^- with different anions. The anion permeability was very similar to the native $GABA_A$ receptor/channel following a sequence of $SCN^- > I^- > NO_3^- > Br^- \geq Cl^-$. Halogenated fatty acids, such as chlorotrifluoroethylene (CTFE) and perfluorinated oligomer acids inhibited the GABA-induced current in oocytes expressing the human retinal GABA ρ_1-subunit or rat brain $GABA_A$ receptor $\alpha_1,\beta_2,\gamma_2$ subunits. The inhibitory effect of halogenated fatty acids demonstrated a carbon chain length-dependent manner of: $C_{10} > C_8 > C_6 > C_4$. Perfluorinated C_8-oligomer acid (PFOA) was less effective at blocking this channel than the C_8-CTFE oligomer acid. Radiolabeled GABA binding assay indicated that CTFE oligomer acids do not interfere at the GABA binding site of the receptor. Furthermore, the C_8-CTFE oligomer fatty acid did not compete with picrotoxin for binding sites within the pore of the channel. These studies demonstrated that the heterologous expression system is useful for studying the molecular interaction between potential neurotoxic agents and neuroreceptors. Our results provide detailed information that should contribute to our understanding of the structure and function of retinal GABA receptors.

Key words: Chlorotrifluoroethylene — GABA receptor — Voltage clamp — Chloride current — *Xenopus* oocyte

Correspondence to: L. Lu

Introduction

γ-Aminobutyric acid (GABA) functions as an inhibitory neurotransmitter in the central nervous system. The $GABA_A$ receptor is the major inhibitory receptor in the brain of vertebrate animals, and functions as a ligand-gated Cl^- conductive channel. Toxicological studies have suggested that the $GABA_A$ receptor is involved in the neurotoxicity of different toxins. The receptor is postulated to be a hetero-oligomeric structure composed of various combinations of at least 5 different subunits: α, β, γ, δ and ρ (Schofield et al., 1987; Olsen & Tobin, 1990; Cutting et al., 1991). Interestingly, each single subunit of the $GABA_A$ receptor demonstrates GABA-activated Cl^- channel activity when expressed in *Xenopus* oocytes (Blair et al., 1988; Pritchett et al., 1988, 1989). Different combinations of $GABA_A$ receptor subunits expressed in the oocytes give different biophysical and pharmacological characteristics, such as varied Cl^- ion conductances and different responses to a variety of ligands, including barbiturate analogues and benzodiazepine (Levitan et al., 1988*a*,*b;* Pritchett et al., 1989; Luddens et al., 1990; Verdoorn et al., 1990). The retinal GABA ρ_1-subunit has been expressed in *Xenopus* oocytes, and forms a GABA ligand-gated Cl^- channel, but its pharmacological properties differ from those of the $GABA_A$ receptor (Cutting et al., 1991; Shimade, Cutting & Uhl, 1992; Kusama et al., 1993). The expressed current has been characterized according to mechanisms of the current activation, desensitization, anion selectivity and sensitive to neurotoxins. This characterized GABA receptor system was used to test for potential neurotoxicity of various chain length halogenated fatty acid metabolites of a newly developed hydraulic fluid.

The perhalogenated hydrocarbon polychlorotrifluoroethylene is a novel nonflammable hydraulic fluid considered for use in military and commercial applications. This hydraulic fluid is composed predominantly

of C_6 and C_8-oligomers of CTFE. These CTFE oligomers are metabolized to corresponding CTFE oligomer acid metabolites by the liver (Brashear, Greene & Mahle, 1992). The hydraulic fluid (DelRaso et al., 1991) and the C_8-CTFE acid metabolite (Kinkead et al., 1987, 1991) have been shown to be metabolized by the liver in rats. Preliminary data indicates that CTFE oligomer fatty acids are present in the brain tissue of exposed rats (*data not reported*). It has previously been shown that certain fatty acids can directly modulate secretory chloride channels in epithelial cells (Hwang, Guggino & Guggino, 1990). Because receptors are involved in many neurotoxic and pharmacologic processes, assays of effects of chemical agents on ligand-gated receptor/channels may be a valid indicator of potential neurotoxicity. Furthermore, the unique blocking pattern of these agents acting on excitable membranes makes electrophysiological techniques ideally suited for studies of the structure of neuroreceptors.

Material and Methods

Synthesis of cRNA for GABA ρ_1-subunit

The full length cDNA (2.05 Kbp) encoding the GABA ρ_1-subunit isolated by the polymerase chain reaction (PCR) from a human eye cDNA library (a generous gift from Dr. Jeremy Nathans) was subcloned into the *BamHI* and *Xho I* sites in the pAlter plasmid (Promega). The cDNA insertion in the pAlter vector was oriented in the T7 to Sp6 direction. The pAlter plasmid with the insert was linearized by the cleavage of template DNAs with the *Xba I* restriction enzyme. $GABA_A$ receptor α_1-, β_2-, and γ_2-subunit were subcloned in pBluescript in the T7 to T3 direction and linearized with *Not I* restriction enzyme. In vitro transcriptions for sense cRNAs were performed in the presence of Cap [$m^7G(5')ppp(5')$] and catalyzed by T7 DNA-dependent RNA polymerase (100 units) at 37°C for two hr. To enhance the production of cRNA transcripts, more polymerase (50 units) was added to the reaction tube during the second hour. Finally, in vitro transcribed RNA was dissolved in DEPC-treated water at a concentration of 1 ng/nl.

Oocyte Preparation and Microinjection

Adult female *Xenopus laevis* frogs (*Xenopus* I, Michigan) were anesthetized by immersion in a 0.15% tricaine methanesulfonate (Ayerst) solution for 20 min. A small incision was made on one side of the abdomen to remove several ovarian lobes. The lobes were torn apart and immersed in a Ca^{2+}-free OR-2 solution (in mM): 100 NaCl, 2 KCl, 1 $MgCl_2$, and 5 Hepes-tris, pH 7.5. Oocytes were defolliculated by incubation with Ca^{2+}-free OR-2 solution containing 2 mg/ml collagenase (Sigma, type 1A) at room temperature (22–24°C) for 2–3 hr. After digestion, oocytes were washed 5 times with OR-2 solution and 5 times with a modified Barth's solution (in mM): MBS, 88 NaCl, 1 KCl, 2.4 $NaHCO_3$, 0.3 $Ca(NO_3)_2$, 0.4 $CaCl_2$, 0.8 $MgSO_4$, 15 Tris-HCl, pH 7.6, and penicillin 100 μg/ml, streptomycin 100 μg/ml). Stage 5–6 oocytes were selected and stored at 18°C in modified Barth's solution. Two days prior to performing the electrophysiology, 50 ng cRNA was injected into oocytes by positive displacement using a 10-μl micropipette.

Two-microelectrode Voltage Clamp

Two-microelectrode voltage clamp experiments were performed in a continuously perfused bath (10 ml/min) at room temperature (22°C). Microelectrodes filled with 3 M KCl with resistances of 1.5 to 2.0 MΩ were made by a horizontal puller (PD-5, Narishige), and were used for delivering current pulses for voltage clamping. The bath was connected through an Ag-AgCl-Agar-3 M-KCl bridge to the voltage-recording amplifier (Axoclamp 2A, Axon Instruments). The data were filtered with a 4-pole Bessel filter at 500 Hz. Voltage pulse protocols and data acquisition were performed by a 486 computer with pCLAMP software (Axon Instruments). Membrane currents were measured in normal Ringers solution or low Cl^- solutions that contained (in mM): 96 NaCl, 2 KCl, 1 $MgCl_2$, 1.2 $CaCl_2$, and 5 Hepes-NaOH, pH 7.4; or 40 NaCl, 56 NaOH, 2 KOH, 1 Mg-gluconate, 1 Ca-gluconate, 5 Hepes, titrated with methanesulfonic acid (Fluka), pH 7.4.

[^{3}H]-GABA Radioligand Binding

Xenopus oocytes were injected with GABA cRNA as indicated above and incubated 72 hr at 18°C. Oocytes (100/treatment group) were then transferred to 10 mL of normal Ringers solution (pH 7.3) and homogenized on ice for 1 min. Aliquots (500 μl; ~1 mg protein) were added to quadruplicate sample tubes per treatment group containing 1.5 mL of normal Ringers. Treatment groups consisted of control, 200 μM and 500 μM C_8-CTFE oligomer acid were added to incubations from 100× stock solutions (10 μl). A second set of tubes containing 2 mM cold GABA in 1.5 ml of normal Ringers was used to determine nonspecific [^{3}H]-GABA binding. Radiolabeled GABA binding was initiated by adding 5 μCi of [^{3}H]-GABA (33.4 Ci/mMol) to all tubes. The tubes were then incubated for 1 hr at room temperature. Incubations were then rapidly vacuum filtered through Whatman GF/B glass fiber filter papers that had been prewetted with ice cold Ringers. Filters were washed 3× with ice-cold Ringers and then placed into plastic scintillation vials. Filters were counted in 5 ml of scintillation cocktail (Scintiverse) in a Beckman counter LS 6000TA. Specific [^{3}H]-GABA activity was determined by subtracting the nonspecific binding.

Chemicals

Gamma-aminobutyric acid (GABA) and picrotoxin (PTX) were purchased from Sigma (St. Louis, MO); Trans-aminocrotonic acid was obtained from Neuramin (Bristol, England); [^{3}H]-GABA (Sp. Act. 33.43 Ci/mmol) was purchased from DuPont NEN (Wilmington, DE); Chlorotrifluoroethylene oligomer acids (C_2–C_{10}) were synthesized by Halocarbo Products (Hackensack, NJ) and 100 mM stock solutions were prepared in ethanol. The perfluorinated octanoic acid was purchased from Aldrich (Milwaukee, WI) and a 100 mM stock solution was prepared in ethanol. All other chemicals were made in buffer stocks and freshly added into solutions before performing experiments.

Results

Basic Properties of Expressed ρ_1-Subunit

The single subunit of the GABA receptor possesses ligand-gated Cl^- channel activity after injection of the

sense cRNA, prepared in vitro using bacteriophage T7 promoter transcription, into *Xenopus* oocytes. Membrane currents were observed in oocytes injected with the ρ_1-subunit cRNA during perfusion of GABA in extracellular solution, but were not observed in the noninjected oocyte. This GABA-activated current was eliminated by washing the GABA agonist from the bath solution. Other control experiments were also performed to confirm that only the sense cRNA-injected oocytes could express the functional receptor/channel protein and that the expressed receptor/channel was activated only by the GABA ligand. The results are as follows: (i) the Cl^- current was not activated by perfusion of 2 mM glycine in the extracellular solution of injected oocytes; (ii) Oocytes injected with antisense cRNAs did not demonstrate any channel activity (*data not shown*). These results indicate that the cloned ρ_1-subunit expressed in oocytes retains the basic characteristics of the GABA-gated channel, and is not a strychnine-sensitive or glycine-activated channel. Perfusion of 20 μM of TACA induced an inward current with a similar amplitude compared to the current induced by 20 μM GABA in the oocyte injected with cRNA. An inward current was activated by 20 μM TACA for 100 sec to reach the saturation level, and then 30 μM GABA was coapplied with TACA during the last 100 sec. The combination of 20 μM TACA and 30 μM GABA had no effect on the current initiated by TACA (Fig. 1*A*). Current-voltage relationships of the GABA-activated current are presented in Fig. 1*B*. The reversal potential measured in the extracellular solution with 120 mM Cl^- was −24 mV and shifted to −13 mV when the extracellular solution was replaced with 40 mM Cl^- (*data not shown*). Anion permeability of the expressed GABA ρ_1-subunit was observed when Cl^- ions (120 mM) in the extracellular bath solution were replaced by 120 mM of different anions (Fig. 1*B*). During anion substitution experiments, current-voltage relationships showed that the reversal potentials were shifted to more positive membrane potentials following a sequence of $SCN^- > NO_3^- > I^- > Br^- \geq Cl^-$ (Table).

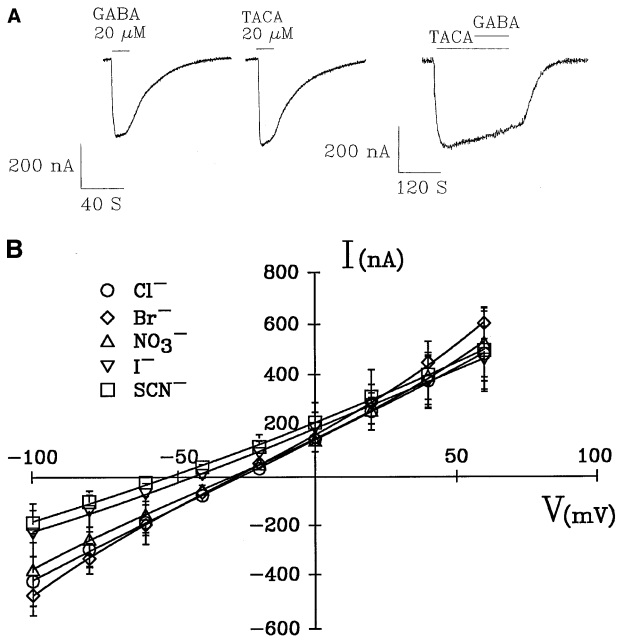

Fig. 1. The newly expressed GABA-activated current in *Xenopus* oocytes injected with synthetic cRNA derived from the full-length cDNA of the ρ_1-subunit. (*A*) An inward current induced by perfusion of 20 μM GABA to compare the current induced by perfusion of 20 μM TACA in the bath solution at membrane potential of −70 mV. An inward current induced by perfusion of 20 μM TACA at a holding potential of −70 mV for 120 sec. During the perfusion of TACA, 30 μM GABA was added into the perfusion line at the end of the first 60 sec to test the integrating effect of TACA and GABA. Dark bars indicate when agonists were perfused into the chamber. (*B*) Relationship between the current and membrane potential. GABA-induced current plotted as a function of membrane potentials to show the anion selectivity of the ρ_1-subunit. The anion selectivity of the ρ_1-subunit was measured by isotonically replacing 120 mM Cl^- ion in the bath with different anions (Br^-, I^-; $NO3^-$, and SCN^-).

Effect of CTFE Oligomer Acids on GABA-activated Currents

The effect of C_8-CTFE oligomer acid ($Cl_{12}F_3C_7COOH$), a halogenated fatty acid, on the expressed current elicited by 10 μM GABA was tested in oocytes injected with the ρ_1-subunit cRNA. At a concentration of 200 μM, C_8-CTFE acid inhibited more than 50% of the GABA-activated current (Fig. 3*A*). The effect of C_8-CTFE oligomer acid on TACA-activated current was also observed in the presence of 10 μM TACA (Fig. 2*A*). C_8-CTFE oligomer acid inhibited both the activation and the steady-state phase of the GABA-activated current, but it had a much stronger effect on the second phase. This indicates that the C_8-CTFE oligomer acid may better interact with the channel when the channel protein is activated by GABA. The inhibitory effect of C_8-CTFE oligomer acid on GABA-activated current was also observed in the oocyte injected with the cRNA transcribed from α_1, β_2 and γ_2 subunits cDNA of $GABA_A$ receptor (Fig. 2*B*). Fractional inhibition of GABA-activated currents (10 μM GABA) was plotted as a function of the C_8-CTFE oligomer acid concentration (Fig. 2*C*). The $GABA_A$ receptor with the combination of α_1, β_2 and γ_2 subunits was also sensitive to the C_8-CTFE oligomer acid (IC_{50} = 72 μM). The effect of C_8-CTFE oligomer acid on the oocyte injected with the ρ_1-subunit cRNA was also examined in the absence of GABA. No current was activated by C_8-CTFE oligomer acid (500 μM) alone (*data not shown*).

Fractional inhibition of GABA-activated current (10 μM GABA) was plotted as a function of concentration of various even chain length CTFE oligomer acids (C_2 to C_{10}) over a dose range of 25 μM to 1 mM, and the half-

Table 1. Anion permeability of GABA ρ_1-subunit

P_A	Cl^-	Br^-	NO_3^-	I^-	SCN^-
$V_{reversal}$ (mV)	−23.8 ± 2.4 (n = 6)	−27.0 ± 3.8 (n = 5)	−34.1 ± 5.6 (n = 5)	−47.0 ± 5.8 (n = 6)	−56.0 ± 7.2 (n = 5)
P_A/P_{Cl}	1.0	1.1	1.5	2.5	3.6

Data are presented as mean ± SE and n represents numbers of each independent experiment.

inhibitory concentration (IC_{50}) was calculated (Fig. 3*A*). The C_{10}-CTFE oligomer acid was found to be the most effective blocker of GABA-activated current with an IC_{50} of 70 μM, followed by the C_8-CTFE oligomer acid (IC_{50} = 175 μM) and finally the C_6-CTFE oligomer acid. An IC_{50} for the C_6-CTFE oligomer acid could not be calculated because the maximal block of the current was found to be ~30%, was reached at a concentration of 500 μM. The C_2 and C_4-CTFE oligomer acids did not have any effect on GABA-activated currents at the highest concentration tested (1.0 mM). Interestingly, a C_8-perfluorinated acid (IC_{50} = 951 μM) was less effective in blocking GABA-activated currents than the C_8-CTFE oligomer acid (Fig. 3*B*). The inhibitory effect of the C_8-CTFE oligomer acid on GABA-activated current was examined at different membrane potentials. The result suggested that the inhibitory effect of the C_8-CTFE oligomer acid was a voltage-independent process (Fig. 3*C*), which indicated that the charge group on the compound was not entering deep enough in the channel pore to sense the electrical field across the membrane.

[^{3}H]-GABA binding assay was also performed to study the interaction between the GABA ligand binding and C_8-CTFE oligomer fatty acid effects on the GABA ρ_1-subunit. Oocytes were separated into four experimental groups (with 100 oocytes in each group): the noninjected oocytes (negative controls), injected oocytes incubated with GABA (positive controls), injected oocytes incubated with GABA plus 200 μM C_8-CTFE oligomer fatty acid, and injected oocytes incubated with GABA plus 500 μM C_8-CTFE oligomer fatty acid. Our results indicated that C_8-CTFE oligomer fatty acid concentrations up to 500 μM had no effect on the binding of [^{3}H]-GABA to its receptor (Fig. 4).

Effects of Toxins on Expressed ρ_1-subunit

It has been shown that GABA-activated Cl^- channels in the brain are sensitive to bicuculline, a competitive inhibitor of GABA, and picrotoxin, a convulsant pore blocker of GABA-activated Cl^- channels. In cRNA of the ρ_1-subunit injected oocytes, the expressed current induced by 5 μM GABA was insensitive to 100 μM bicuculline (*data not shown*). GABA-activated current was inhibited 80% by the introduction of 4 μM picrotoxin into the bath solution (Fig. 5*A*). This picrotoxin inhibition was reversible by washing with normal Ringers solution for 5 min (*data not shown*). A concentration-response relation curve was obtained by perfusion with different concentrations of picrotoxin (Fig. 5*A*). IC_{50} of picrotoxin block was 1.6 ± 0.3 μM (n = 4) in the presence of 3 μM GABA ligand. Furthermore, the interaction of picrotoxin and C_8-CTFE oligomer fatty acid was examined in injected oocytes. C_8-CTFE oligomer fatty acid (200 μM) had no effect on picrotoxin (10 μM)-induced block of GABA-activated Cl^- currents (Fig. 5*B*). A 30-sec exposure of oocytes to 10 μM picrotoxin in the absence of GABA had no effect on the degree of channel blocking induced by a solution containing picrotoxin, C_8-CTFE oligomer fatty acid and GABA. However, a 30-sec exposure of oocytes to C_8-CTFE oligomer fatty acid in the absence of GABA resulted in a 2-fold enhanced blocking of the GABA-induced current by the solution containing picrotoxin, C_8-CTFE and GABA (*data not shown*).

Discussion

A single GABA ρ_1-subunit and the combination of α, β and γ subunits of $GABA_A$ receptor have been expressed in the membrane of *Xenopus* oocytes to form functional GABA-activated anion channels following injection with cRNAs coding for these receptor/channel subunits. Heterologously expressed GABA ρ_1-subunit in *Xenopus* oocytes are activated by GABA and TACA at 10^{-6} M concentration. The current initiated by TACA at a near saturation dose was not further activated by addition of a saturation dose of GABA in injected oocytes (Fig. 1*A*). This result indicates that TACA and GABA may affect the same site of the receptor domain of the ρ_1-subunit. The change of the reversal potential measured in different extracellular Cl^- concentrations was consistent with chemical equilibrium potentials calculated from the Nernst equation for the Cl^- ion (estimated intracellular Cl^- concentration for oocytes is 30 mM) (Barish, 1983). The selectivity sequence of the permeability to anions for the ρ_1-subunit (Table) was consistent with the sequence observed in brain $GABA_A$ receptors (Hamill, Boorman & Sakmann, 1981; Bormann, Hamill & Sakmann, 1987; Levitan et al., 1988).

Many antagonists (competitive inhibitors) of GABA inhibit the GABA-induced Cl^- current in neurons. Rep-

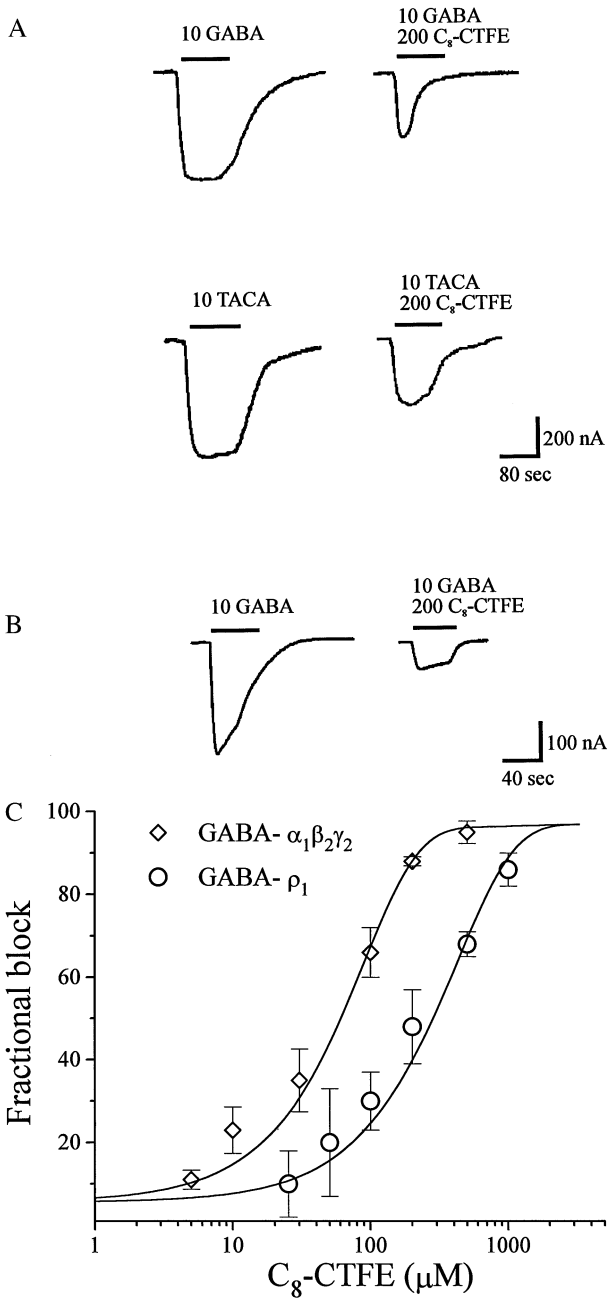

Fig. 2. Effect of C_8-CTFE oligomer fatty acid on GABA-activated currents. (*A*) Inhibitory effect of C_8-CTFE oligomer fatty acid on GABA- or TACA-activated current in the ρ_1-subunit injected oocytes. Inward current activated by perfusion of 10 μM GABA at a membrane potential of −70 mV and inhibited more than 60% of the peak current by the addition of 200 μM C_8-CTFE oligomer fatty acid. Inward current activated by perfusion of 10 μM TACA and inhibited approximately 30% by the addition of 200 μM C_8-CTFE oligomer fatty acid in the perfusion bath at a holding potential of −70 mV. (*B*) Inhibitory effect of C_8-CTFE oligomer fatty acid on GABA-activated current in the oocyte injected with $GABA_A$ receptor α, β and γ subunits. Whole-cell current activated by 10 μM GABA at the membrane potential of −70 mV was blocked by 200 μM C_8-CTFE oligomer fatty acid. (*C*) Fractional block plotted as a function of the concentration of C_8-CTFE oligomer fatty acid. Circles and diamonds in dose-response curves represent oocytes expressing GABA ρ_1-subunit and the combination of GABA α_1, β_2, and γ_2 subunits, respectively. Data points with SE bars were collected from 4–7 independent experiments and fit by the dose-dependent saturation function.

resentative of these antagonists is bicuculline (Curtis et al., 1971; Barker & Mathers, 1981; Jackson et al., 1982). The expressed ρ_1-subunit was insensitive to bicuculline. Our observation is consistent with the results from patch clamp studies in bipolar cells of rat retina (Feigenspan, Wassle & Bormann, 1993) and in horizontal cells of fish retina (Qian & Dowling, 1993). This probably explains why the retinal GABA receptor/channel is pharmacologically distinct from brain $GABA_A$ receptors. It has been suggested that the ρ_1-subunit belongs to the $GABA_C$ class (Drew, Johnston & Weatherby, 1984; Shimada et al., 1992). A number of drugs either increase or decrease the GABA-activated Cl^- current. Barbiturates (like pentobarbitone), anxiolytic benzodiazepines (diazepan) and alcohol increase the whole-cell current induced by GABA and prolong the single GABA Cl^- channel burst duration (Study & Barker, 1981; Wafford et al., 1990; Proctor et al., 1992). Other drugs, such as penicillin G and pentylenetetrazol, and convulsant drugs like picrotoxin and t-butylbicyclophosphorothionate (TBPS), reduce the GABA-activated Cl^- current and inhibit channel activity. These drugs are thought to inhibit the receptor Cl^- channel by directly blocking the channel pore (Akaike et al., 1985) or by an unknown mechanism. It is of interest to determine the differences in the roles that GABA receptor/channel play in the retina and in the inhibitory pathway in the brain; thus, the interaction between the GABA receptor/channel and various drugs is becoming an important topic for future studies. A series of experiments were performed to test the effects of barbiturate and benzodiazepine drugs on the expressed ρ_1-subunit (*data not shown*). Our results were similar to previous studies, which indicated that neither drug had a significant effect on the channel (Shimada et al., 1992). It is known that GABA-activated Cl^- channels are sensitive to picrotoxin, a convulsant inhibitor. The GABA ρ_1-subunit was inhibited approximately 80% by the addition of 4 μM picrotoxin (Fig. 5*A*, inserted trace) in the presence of 3 μM GABA (IC_{50} = 1.6 μM). This picrotoxin inhibition was reversible. The ρ_1-subunit was highly sensitive to picrotoxin compared to other GABA-activated Cl^- channels that have been expressed in oocytes. The completely inhibitory concentration of picrotoxin from brain $GABA_A$ subunits expressed in oocytes ranges between 5 μM and 500 μM (Blair et al., 1988; Levitan et al., 1988).

The C_8-CTFE oligomer fatty acid used in the present study is a recently identified metabolite of a novel hydraulic fluid (3.1 oil) composed primarily of CTFE de-

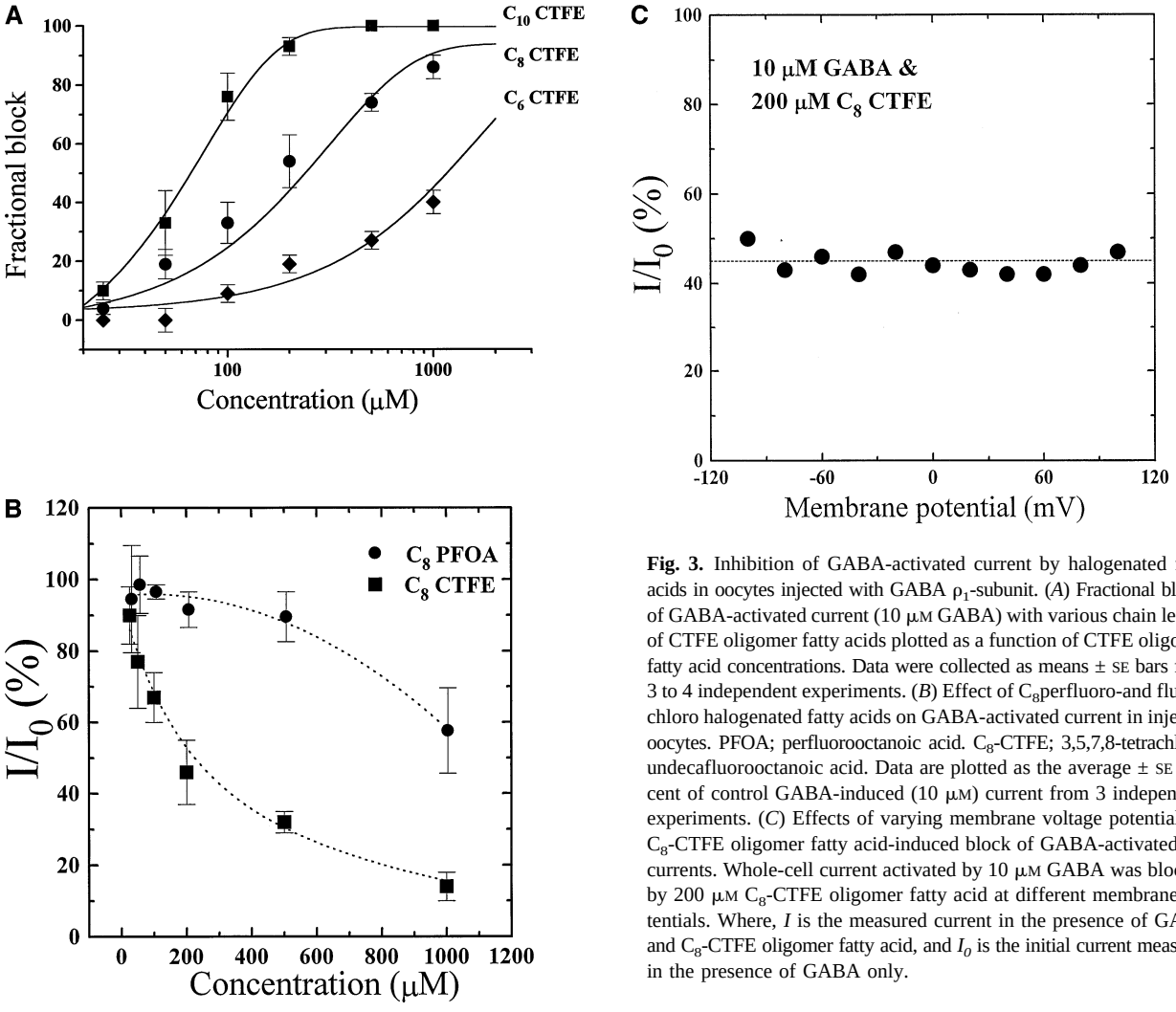

Fig. 3. Inhibition of GABA-activated current by halogenated fatty acids in oocytes injected with GABA ρ_1-subunit. (*A*) Fractional blocks of GABA-activated current (10 μM GABA) with various chain length of CTFE oligomer fatty acids plotted as a function of CTFE oligomer fatty acid concentrations. Data were collected as means ± SE bars from 3 to 4 independent experiments. (*B*) Effect of C_8perfluoro-and fluoro/chloro halogenated fatty acids on GABA-activated current in injected oocytes. PFOA; perfluorooctanoic acid. C_8-CTFE; 3,5,7,8-tetrachloroundecafluorooctanoic acid. Data are plotted as the average ± SE percent of control GABA-induced (10 μM) current from 3 independent experiments. (*C*) Effects of varying membrane voltage potentials of C_8-CTFE oligomer fatty acid-induced block of GABA-activated Cl^- currents. Whole-cell current activated by 10 μM GABA was blocked by 200 μM C_8-CTFE oligomer fatty acid at different membrane potentials. Where, *I* is the measured current in the presence of GABA and C_8-CTFE oligomer fatty acid, and I_0 is the initial current measured in the presence of GABA only.

rived C_6 and C_8 oligomers (DelRaso et al., 1991; Brashear et al., 1992). This observation suggested that neurological effects may have resulted from exposure to the C_8-CTFE oligomer. This is partially supported by the finding that the C_8-CTFE oligomer fatty acid was a more potent blocker of the GABA-activated channel currents than the C_6-CTFE oligomer fatty acid (Fig. 3*A*). Furthermore, it was found that a C_{10}-CTFE oligomer fatty acid was even more potent than the C_8-CTFE oligomer fatty acid in blocking these channel currents. These results suggest that the potency of GABA-activated channel current block is dependent on CTFE oligomer acid chain length.

Interestingly, a C_8-perfluorinated fatty acid was not as effective at blocking GABA-activated channel currents as the C_8-CTFE oligomer fatty acid (Fig. 3*B*). This suggests that chlorine atoms on the halogenated hydrocarbon fatty acid allow for additional interactions of the CTFE oligomer fatty acid with other sites on the channel. [^{3}H]-GABA binding studies indicated that the C_8-CTFE oligomer fatty acid does not bind at the GABA binding site (Fig. 4), nor at the picrotoxin binding site within the channel pore (Fig. 5*B*). Based on the observations above, we can speculate a model of GABA-activated Cl^- channel inhibition by CTFE oligomer acids where the acid binds reversibly at the mouth region of the channel pore and that chlorine atoms on these halogenated fatty acids are responsible for attracting the hydrocarbon chain into the pore, of the channel where Cl^- ion pathway exists.

The model is partially supported by the finding that pretreatment of oocytes with the C_8-CTFE oligomer fatty acid, and not picrotoxin, resulted in enhanced blocking of GABA-activated channels by a solution containing the two toxins in the presence of GABA. Thus, this effect could be explained by the binding of halogenated fatty acid to the channel in the closed state, but blocking the open state of the channel. Perfusion of picrotoxin 30 sec prior to coapplications of picrotoxin, CTFE, and GABA did not induce an additional inhibition of the GABA-

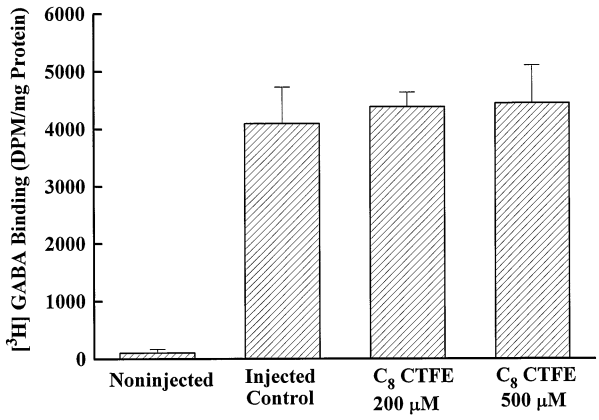

Fig. 4. Ligand binding assay of interaction between [^{3}H]-labeled GABA and C_8-CTFE oligomer fatty acid in oocytes injected with cRNA of the GABA ρ_1-subunit. Hatched bars represent radioactive counts normalized by the protein weight in mg. Values for the control, noninjected oocytes, injected oocyte, injected oocytes with 200 μM C_8-CTFE oligomer fatty acid, and injected oocytes with 500 μM C_8-CTFE oligomer fatty acid were 113 ± 56 DPM/mg, 4102 ± 625 DPM/mg, 4391 ± 246 DPM/mg and 4456 ± 649, respectively. There was significant difference between the noninjected group and injected groups ($P < 0.005$), but there was no significance among those injected groups ($P > 0.5$).

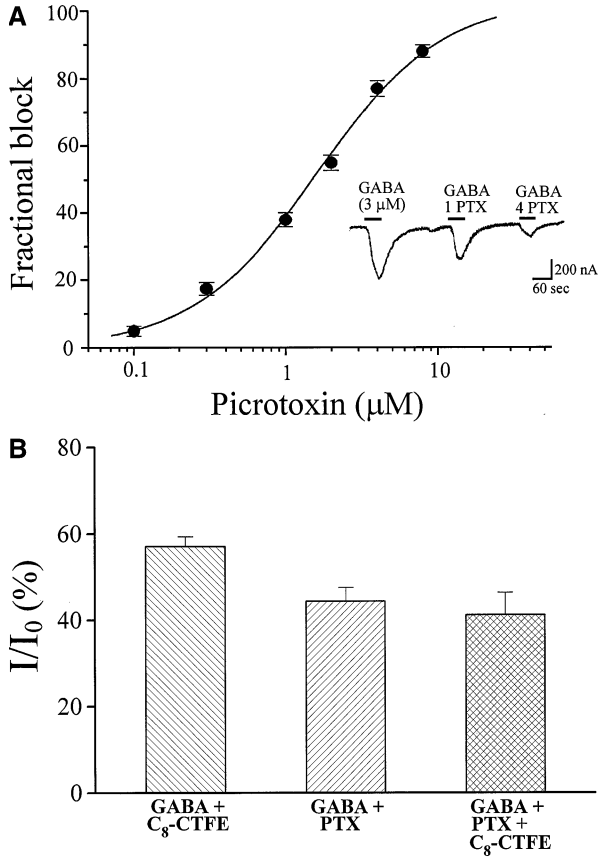

Fig. 5. Interaction of picrotoxin (PTX) and C_8-CTFE oligomer fatty acid in inhibition of the GABA-activated current by the ρ_1-subunit injected oocytes. (*A*) Fractional block of the GABA-activated current plotted as a function of PTX concentrations. Data were collected as means ± SE bars (a few SE bars not visible well in the figure) from 4 independent experiments and fitted by the single binding saturation function. The insertion trance was activated by 3 μM GABA and blocked by 1 μM and 4 μM PTX. (*B*) Effect of C_8-CTFE oligomer fatty acid on PTX block of GABA-activated current. Bars represent the average percent-of-control ± SE ($n = 3$) of 10 μM GABA-activated current. Exposure of injected oocytes to the two test agents was initiated by perfusion of a normal Ringers solution containing the C_8-CTFE oligomer fatty acid, PTX and 10 μM GABA.

activated current. The possible interpretation for this is that the blocking site for CTFE oligomer fatty acid is deeper in the channel pore than the picrotoxin site because it has been shown that picrotoxin blocks GABA receptor/channel by interacting with the channel pore region. Because the pore region of the channel is inaccessible to the tail of the fatty acid in the closed state or in the presence picrotoxin, the fatty acid tail may be free to move about the mouth region of the channel, thus diminishing the inhibitory effect of the fatty acid. Another possibility, but unlikely, is that binding of the halogenated fatty acid induced a conformational change that makes the coupling process between GABA binding and channel opening less efficient.

This would explain why longer chain CTFE oligomer fatty acids are more potent GABA channel blockers. Longer chain halogenated fatty acids containing chlorine atoms would be attracted deeper into the channel pore and physically plug the pore preventing the passage of Cl^- ions. Although the exact mechanism of inhibition of GABA-activated channel currents by these halogenated fatty acids is not known, it has been previously shown that arachidonic and other *cis* unsaturated fatty acids can directly modulate chloride channels (Hwang et al., 1990). It is possible that these halogenated fatty acids inhibit the activity of GABA activated current by inserting into the membrane in close proximity to the channel, but this is unlikely because the C_8-perfluorinated fatty acid did not produce the same effect as the C_8-CTFE oligomer fatty acid.

In conclusion, a GABA-activated and Cl^--selective channel was characterized in the membrane of *Xenopus* oocytes after injection of the cRNA coding for the human retinal GABA receptor ρ_1-subunit. Compared with other GABA-activated Cl^- channels that have been expressed in oocytes, the expressed ρ_1-subunit has many similarities to $GABA_A$ receptor subunits from the brain. The Cl^- channel is permeable to other anions, and is blocked by picrotoxin. However, other pharmacological properties of the retinal GABA receptor ρ_1-subunit are quite distinct from other GABA receptor subunits from the brain. In addition, the long chain halogenated fatty acids ($\geqslant C_6$) were found to block the Cl^- permeability of the GABA channel. Because a preliminary study has

found that the C_6 and C_8-CTFE oligomer fatty acid metabolites of poly-CTFE hydraulic oil are present in the brain tissue of exposed rats (*data not reported*), these fatty acid metabolites are potentially neurotoxic. Our data indicate that the use of a *Xenopus* oocyte expression system for GABA receptors may be useful as an in vitro model for receptor-ligand mediated neurotoxicity that will allow for the reduction of animal usage in neurotoxicity testing.

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References

Akaike, N., Hattori, K., Oomura, Y., Carpenter, D.O. 1985. Bicuculline and picrotoxin block gamma-aminobutyric acid-gated Cl^- conductance by different mechanisms. *Experientia* **41:**70–71

Barish, M. 1983. A Transient calcium-dependent chloride current in the immature *Xenopus* oocyte. *J. Physiol.* **342:**309–325

Barker, J.L., Mathers, D.A. 1981. GABA analogues activate channels of different duration on cultured mouse spinal neurons. *Science* **212:** 358–361

Blair, L.A.C., Levitan, E.S., Marshall, J., Dionne, V.E., Barnard, E.A. 1988. Single Subunits of the $GABA_A$ receptor from ion channels with properties of the native receptor. *Science* **242:**577–579

Bormann, J., Hamill, O.P., Sakmann, B. 1987. Mechanism of anion permeation through channels gated by glycine and gamma-aminobutyric acid in mouse cultured spinal neurons. *J. Physiol.* **385:**243–286

Brashear, W.T., Greene, R.J., Mahle, D.A. 1992. Structural determination of the carboxylic acid metabolites of polychlorotrifluoroethylene. *Xenobiotica* **22:**499–506

Curtis, D.R., Duggan, A.W., Felix, D., Johnston, G.A.R. 1971. The specificity of strychnine as a glycine antagonist in the mammalian spinal cord. *Brain Res.* **32:**69–96

Cutting, G., Lu, L., O'Hara, B., Kasch, L.M., Donovan, D., Antonarakis, S.E., Guggino, W.B., Uhl, G.R., Kazazian, H.H. Jr. 1991. Cloning of the γ-aminobutyric acid (GABA) Rho-1 cDNA: a GABA receptor subunit highly expressed in the retina. *Proc. Natl. Acad. Sci. USA* **88:**2673–2677

DelRaso, N.J., Godin, C.S., Jones, C.E., Wall, H.G., Mattie, D.R., Flemming, C.D. 1991. Comparative hepatotoxicity of two polychlorotrifluoroethylenes (3.1 oils) and two chlorotrifluoroethylene (CTFE) oligomers in male Fischer 344 rats. *Fundamental and Applied Toxicology* **17:**550–562

Drew, C.A., Johnston, G.A.R., Weatherby, R.P. 1984. Bicuculline-insensitive GABA receptors: studies on the binding of (−)-baclofen to rat cerebellar membranes. *Neurosci. Letters* **62:**317–321

Feigenspan, A., Wassle, H., Bormann, J. 1993. Pharmacology of GABA receptor Cl^- channels in rat retinal bipolar cells. *Nature* **361:**159–161

Hamill, O.P., Bormann, J., Sakmann, B. 1983. Activation of multiple-conductance state chloride channels in spinal neurons by glycine and GABA. *Nature* **305:**805–808

Hwang, T.C., Guggino, S.E., Guggino, W.B. 1990. Direct modulation of secretory chloride channels by arachidonic and other *cis* unsaturated fatty acids. *Proc. Natl. Acad. Sci. USA* **87:**5706–5709

Jackson, M.B., Lecar, H., Mathers, D.A., Barker, J.L. 1982. Single channel currents activated by gamma-aminobutyric acid, muscimol, and (−)-pentobarbital in cultured spinal neurons. *J. Neurosci.* **2:**889–894

Kinkead, E.r., Bunger, S.K., Wolfe, R.E., Flemming, C.D., Whitmire, R.E., Wall, H.G. 1991. Repeated-dose gavage studies on polychlorotrifluoroethylene acids. *Toxicol. Indust. Health* **7:**295–307

Kinkead, E.R., Gaworski, C.L., Horton, J.R., Boosinger, T.R. 1987. Chlorotrifluoroethylene oligomer: evaluation of acute delayed neurotoxicity in hens, and study of absorption and metabolism in rats following oral, dermal, and inhalation exposure. AAMRL-TR-87-044

Kusama, T., Wang, T-L, Guggino, W.B., Cutting, G.R., Uhl, G.R. 1993. GABA ρ_2 receptor pharmacological profile: GABA recognition site similarities to ρ_1. *Euro. J. Pharmacol.* **245:**83–84

Levitan, E.S., Blair, L.A.C., Dionne, V.E., Barnard, E.A. 1988*a*. Biophysical and pharmacological properties of cloned $GABA_A$ receptor subunits expressed in *Xenopus* oocytes. *Neuron* **1:**773–781

Levitan, E.S., Schofield, P.R., Burt, D.R., Rhee, L.M., Wisden, W., Kohler, M., Fujita, N., Rodriguez, H.E., Stephenson, A., Darlison, M.G., Barnard, E.A., Seeburg, P.H. 1988*b*. Structural and functional basis for $GABA_A$ receptor heterogeneity. *Nature* **335:**76–79

Luddens, H., Pritchett, D.B., Kohler, M., Killish, I., Keinanen, K., Monyer, H., Sprengel, R., Seeburg, P.H. 1990. Cerebellar $GABA_A$ receptor selective for a behavioral alcohol antagonist. *Nature* **346:**648–651

Olsen, R.W., Tobin, A.J. 1990. Molecular biology of $GABA_A$ receptor. *FASEB J.* **4:**1469–1480

Pritchett, D.B., Sontheimer, H., Gorman, C.M., Kettermann, H., Seeburg, P.H., Schofield, P.R. 1988. Transient expression shows ligand gating and allosteric potentiation of $GABA_A$ receptor subunits. *Science* **242:**1306–1308

Pritchett, D.B., Sonthemimer, H., Shivers, B.D., Ymer, S., Kettermann, H., Schofield, P.R., Seeburg, P.H. 1989. Importance of a novel $GABA_A$ receptor subunit for benzodiazepine pharmacology. *Nature* **338:**582–585

Proctor, W.R., Soldo, B.L., Allan, A.M., Dunwiddie, T.V. 1992. Ethanol enhances synaptically evoked $GABA_A$ receptor-mediated responses in cerebral cortical neurons in rat brain slices. *Brain Res.* **595:**220–227

Qian, H., Dowling, J.E. 1993. Novel GABA responses from rod-driven retinal horizontal cells. *Nature* **361:**162–164

Schofield, P.R., Darlison, M.G., Fujita, N., Burt, D.R., Stephenson, F.A., Rodriguez, H., Rhee, L.M., Ramachandran, J., Reale, V., Glencorse, T.A., Seeburg, P.H., Barnard, E.A. 1987. Sequence and functional expression of the $GABA_A$ receptor shows a ligand-gated receptor super-family. *Nature* **328:**221–227

Shimada, S.G., Cutting, G., Uhl, G.R. 1992. γ-aminobutyric acid A or C receptor? γ-aminobutyric acid ρ_1 receptor RNA induces bicuculline-, barbiturate-, and benzodiazepine-insensitive γ-aminobutyric acid responses in *Xenopus* oocytes. *Mol. Pharmacol.* **41:**683–687

Study, R.E., Barker, J.L. 1981. Diazepam and (−)-pentobarbital: fluctuation analysis reveals different mechanisms of gamma-aminobutyric acid responses in cultured central neurons. *Proc. Natl. Acad. Sci. USA* **78:**7180–7184

Verdoorn, T.A., Draghun, A., Ymer, S., Seeburg, P.H., Sakmann, B. 1990. Functional properties of recombinant rat $GABA_A$ receptors depend upon subunit composition. *Neuron* **4:**919–928

Wafford, K.A., Burnett, D.M., Dunwiddie, T.V., Harris, R.A. 1990. Genetic differences in the Ethanol sensitivity of $GABA_A$ receptors expressed in *Xenopus* oocytes. *Science* **249:**291–293