0026-895X/96/041024-07$3.00/0

MOLECULAR PHARMACOLOGY, 50:1024–1030 (1996).

Design and *In Vitro* Pharmacology of a Selective γ-Aminobutyric Acid$_C$ Receptor Antagonist

D. RAGOZZINO, R. M. WOODWARD, Y. MURATA, F. EUSEBI, L. E. OVERMAN, and R. MILEDI

Centroricerca Sperimentale Instituto Regina Elena, I-00158 Rome, Italy (D.R., F.E.), Acea Pharmaceuticals, Irvine, California 92718 (R.M.W.), and Departments of Chemistry (Y.M., L.O.) and Psychobiology (R.M.), University of California, Irvine, California 92717-4550

Received May 13, 1996; Accepted July 2, 1996

SUMMARY

In mammals, receptors for the inhibitory neurotransmitter γ-aminobutyric acid (GABA) are divided into three pharmacological classes, which are denoted GABA$_A$, GABA$_B$, and GABA$_C$. GABA$_C$ receptors are defined by their insensitivity to the GABA$_A$ receptor antagonist bicuculline and the GABA$_B$ receptor agonist (−)-baclofen. GABA$_C$ receptors probably are a heterogeneous group of proteins. The most extensively studied mammalian GABA$_C$ receptors are those found in neurons of the outer retina. These receptors are GABA-gated Cl^- channels comprised of ρ subunits, of which there are two subtypes. The physiological functions served by GABA$_C$ receptors are largely unknown; to determine the functions, it would be useful to have GABA$_C$-selective ligands. In a previous study, we found that isoguvacine, a GABA$_A$-selective agonist, and 3-aminopropyl-(methyl)phosphinic acid (3-APMPA), a GABA$_B$-selective agonist, show affinity for retinal GABA$_C$ receptors. In particular, 3-APMPA is an antagonist with low micromolar potency ($K_b \simeq 1$ μM). Here, we report the synthesis and pharmacological characterization of (1,2,5,6-tetrahydropyridine-4-yl)methylphosphinic acid (TPMPA), a hybrid of isoguvacine and 3-APMPA designed to retain affinity for GABA$_C$ receptors but not to interact with GABA$_A$ or GABA$_B$ receptors. Electrical assays show that TPMPA is a competitive antagonist of cloned human ρ1 GABA$_C$ receptors expressed in *Xenopus laevis* oocytes ($K_b \simeq 2$ μM). TPMPA is >100-fold weaker as an inhibitor of rat brain GABA$_A$ receptors expressed in oocytes ($K_b \simeq 320$ μM) and has only weak agonist activity on GABA$_B$ receptors assayed in rat hippocampal slices ($EC_{50} \simeq 500$ μM). TPMPA should be a useful pharmacological probe with which to investigate GABA$_C$ receptor function in the outer retina and in any other areas of the nervous system in which these types of receptor are present.

Mammalian GABA receptors fall into three pharmacological classes: GABA$_A$, GABA$_B$ and GABA$_C$ (for reviews, see Refs. 1–3). GABA$_C$ receptors are defined by insensitivity to the GABA$_A$-selective antagonist bicuculline and to the GABA$_B$-selective agonist baclofen (3–5). Evidence suggests that there may be more than one type of receptor that falls into this category (3, 4). Currently, the only GABA$_C$ receptors with known molecular composition are those found in retinal neurons. Retinal GABA$_C$ receptors are ligand-gated Cl^- channels formed by homo-oligomeric or hetero-oligomeric assembly of GABA ρ1 and ρ2 subunits (6, 7). The ρ1 subunits are probably restricted to the retina (6, 8). The ρ2 subunits, on the other hand, have been detected in various regions of the nervous system (8). Interestingly, ρ1 GABA$_C$ receptors can also be activated by the inhibitory neurotransmitter glycine (9, 10), either directly (in the millimolar concentration range) or in combination with GABA (in the micromolar concentration range).

This work was supported by United States Public Health Service Grants MH48358 (R.M.W., R.M.) and HL25854 (Y.M., L.E.O.).

The pharmacology and biophysics of mammalian retinal GABA$_C$ receptors have been studied in detail with receptors expressed in *Xenopus laevis* oocytes by retinal poly(A)$^+$ RNA (11–15), with cloned ρ1 and ρ2 subunits expressed in oocytes and COS-7 cells (16–19), and with native receptors in retinal neurons (20, 21). Receptor properties generally show good agreement among these different assay systems. In addition, receptors with similar properties and pharmacology have been characterized in fish retinal neurons (22).

The physiological functions of ρ1 and ρ2 GABA$_C$ receptors remain uncertain. Their localization in retinal neurons suggests that they may be involved in lateral inhibition of light-induced responses (20–23). Progress in investigating the function and subcellular localization of GABA$_C$ receptors in retina and other regions of the brain has been hampered by a paucity of subtype-specific ligands.

Previous studies had given insights into how GABA$_C$-selective antagonists might be designed (15, 17, 18). In particular, we had found that 3-APMPA, a high-potency GABA$_B$ receptor agonist (24), is a competitive antagonist of retinal

ABBREVIATIONS: GABA, γ-aminobutyric acid; 3-APMPA, 3-aminopropyl(methyl)phosphinic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TPMPA, (1,2,5,6-tetrahydropyridine-4-yl)methylphosphinic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

$GABA_C$ receptors ($K_b \simeq 1$ μM) and is inactive as a ligand for $GABA_A$ receptors (15, 25). In terms of structure, 3-APMPA is a flexible GABA analogue in which the carboxyl group has been substituted with a methylphosphinic acid moiety (Fig. 1). Thus, the methylphosphinic acid substitution already confers moderate antagonist potency for $GABA_C$ receptors and selectivity with respect to $GABA_A$ receptors. Using 3-APMPA as a starting point, the problem to overcome when designing a $GABA_C$-selective ligand was to obviate interactions at $GABA_B$ receptors. We reasoned that incorporation of the methylphosphinic acid group into another GABA analogue that is inactive at $GABA_B$ receptors might be a way of solving this problem. One candidate is the $GABA_A$ agonist isoguvacine (15). This compound is a semirigid analogue of GABA in which the amino group has been incorporated into a tetrahydropyridine ring structure (Fig. 1). Isoguvacine is a weak agonist for retinal $GABA_C$ receptors (15, 17) but has the desired property of being inactive at $GABA_B$ receptors (37). We therefore synthesized TPMPA, the methylphosphinic acid analogue of isoguvacine (Fig. 1). In the current study, we describe the *in vitro* pharmacology of this compound.

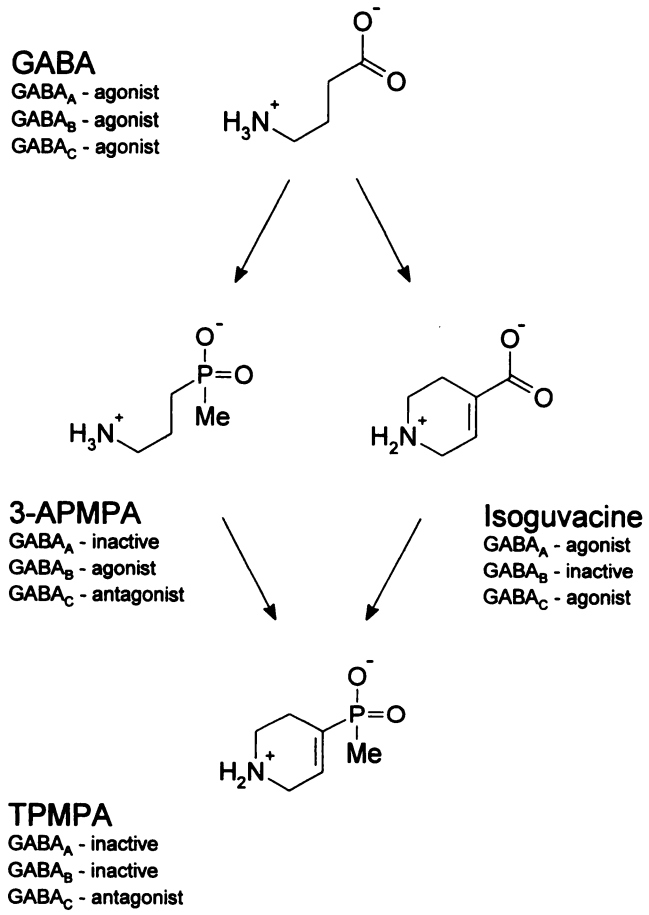

Fig. 1. Structures and pharmacology of GABA, isoguvacine, 3-APMPA, and proposed pharmacology of TPMPA: strategy for designing a $GABA_C$-selective ligand. *Inactive*, relative potency with respect to other receptor subtypes rather than an absolute measure of activity.

Materials and Methods

Synthesis of TPMPA. TPMPA was synthesized in three steps from 1-benzyl-4-iodo-1,2,5,6-tetrahydropyridine (27). First, the vinyl iodide was coupled with methoxymethylphosphonite in the presence of $Pd(PPh_3)_4$ to afford the corresponding vinyl phosphinate (28, 29). The benzyl-protecting group was then cleaved by sequential treatment with 2(trimethylsilyl)ethyl chloroformate and HBr/OHAc (30), and the resulting product was purified by ion exchange chromatography (Dowex 50W) to provide TPMPA. Details of the synthesis are published elsewhere (46).

The *X. laevis* oocyte expression system. Poly(A)$^+$ RNA was isolated from rat cerebral cortex using the phenol/chloroform method according to Woodward *et al.* (12). Preparation of cRNA encoding the human ρ1 receptor subunit was performed according to Calvo *et al.* (19). General procedures for preparation and microinjection of oocytes were performed according to Hawkinson *et al.* (31) and Miledi *et al.* (32). Briefly, frogs were anesthetized (30–60 min) with 0.15% 3-aminobenzoic acid ethyl ester (MS-222). A small portion of the ovary was surgically removed, and mature oocytes were dissected while still surrounded by their enveloping layers. Oocytes were stored in Barth's medium (containing 88 mM NaCl, 1 mM KCl, 0.41 mM $CaCl_2$, 0.33 mM $Ca(NO_3)_2$, 0.82 mM $MgSO_4$, 2.4 mM $NaHCO_3$, 5 mM HEPES, pH 7.4, with 0.1 mg/ml gentamycin sulfate). Tissue surrounding oocytes, except for the vitelline layer, was removed enzymatically by treatment with collagenase (0.5 mg/ml, 45–75 min; Boehringer-Mannheim Biochemicals, Indianapolis, IN). Denuded oocytes were injected with ~10 ng of cRNA encoding the rat GABA ρ1 receptor subunit or with ~50 ng of rat cerebral cortex poly(A)$^+$ RNA. Electrical recordings were made using Dagan TEV-200 or homemade voltage clamps in frog Ringer's solution (containing 115 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 5 mM HEPES, pH 7.4). Drug and wash solutions were applied to oocytes by bath perfusion or using a multibarreled linear array of capillary tubes mounted to a micromanipulator (31). The tip of the "active" capillary was positioned 200–500 μm from the oocyte surface, and the flow rate (5–10 ml/min) was such that the solution surrounding the oocyte was completely dependent on flow from that tube.

Rat hippocampal slices. Hippocampal slices were prepared from 7–14-day-old rats according to Edwards *et al.* (33). Briefly, ACI/T rats were anesthetized with ether and killed by decapitation. The brain was quickly removed and placed into ice-cold saline buffer (125 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, 10 mM glucose 10, equilibrated at pH 7.3 with 95% O_2/5% CO_2). The hippocampus was then partially dissected and glued onto the stage of a vibrating Microslicer (Dosaka, Kyoto, Japan). Transverse 200–250-μm-thick hippocampal slices were cut in ice-cold oxygenated saline. Slices were maintained at room temperature for 1–8 hr and then transferred to an upright microscope (Axioscope, Zeiss, Oberkochen, Germany) with 640× Nomarski optics. During experiments, the slices were maintained at room temperature (21–25°) and superfused with oxygenated saline (1.5–2.0 ml/min). All drugs were applied by bath perfusion.

Whole-cell pipettes were pulled from borosilicate glass (1.7–1.1 mm; Hilgenberg, Maisfield, Germany) using a two-stage puller (List Medical, Darmstadt, Germany) and fire polished to a final resistance of 2–4 MΩ. Pipettes were filled with intracellular solution (140 mM KCl, 1 mM $CaCl_2$, 4 mM $MgCl_2$, 10 mM HEPES, 11 mM EGTA, 2 mMNa-ATP, equilibrated at pH 7.3 with KOH). In some experiments, KCl was substituted with K-gluconate (140 mM K-gluconate, 10 mM KCl) and 5 mM creatine phosphate. All solutions were adjusted to a final osmolarity of ~290 mOsM. Membrane currents were recorded with an Axopatch 200A (Axon Instruments, Burlingame, CA), filtered at 2 kHz, and data analyzed using pClamp software (Axon Instruments).

Excitatory synaptic currents were elicited by stimulating (0.1 Hz) the Schaffer collateral-commissural fibers in the stratum radiatum (5–50 V, 50–300 μsec) with a bipolar electrode fabricated from a

theta glass tube and filled with external solution. Remote platinum wires were connected via an isolated stimulus unit (Grass Instruments, Quincy, MA). $GABA_A$ receptors were blocked by the addition of 20 μM bicuculline methiodide to the superfusing solution. Uncompensated series resistance measured ~10 MΩ. The voltage error due to the combined flow of recorded synaptic currents (<200 pA) and leak currents through the resistance was estimated to be <5 mV.

Data analysis. Data were analyzed as described in Woodward *et al* (34). Briefly, data for GABA concentration-response relations were fit to equation 1 (SigmaPlot, Jandel Scientific, San Rafael, CA):

$$\frac{I}{I_{max}} = \frac{1}{1 + (10^{-pEC_{50}}/[\text{agonist}])^n} \quad (1)$$

where I is the measured response, I_{max} is the maximum GABA response, n is the slope factor, and pEC_{50} is $-\log EC_{50}$, where EC_{50} is the agonist concentration that produces a half-maximum response. Concentration-inhibition curves for hippocampal slice experiments were similarly fit using the logistic equation. Apparent antagonist dissociation constants (K_b values) for TPMPA were determined from a simultaneous fit of concentration-response data in the presence and absence of inhibitor using equation 2.

$$\frac{I}{I_{max}} = \frac{1}{1 + \left\{\frac{10^{-pEC_{50}}\left(\frac{1 + [\text{antagonist}]}{10^{-pK_b}}\right)}{[\text{agonist}]}\right\}^n} \quad (2)$$

where [antagonist] is the fixed concentration of antagonist, and pK_b is $-\log K_b$. Statistical conformity to the simple competitive model was tested by F test (34). F values in the text are given in the form $F_{(df1-df2)/df1}$, where $df1$ and $df2$ are the degrees of freedom for individual fits and simultaneous fits, respectively. The values for p given in the text were calculated using Student's t test.

Drugs. 3-APMPA was obtained from Tocris Cookson (St. Louis, MO). Other drugs were from Sigma Chemical Co. (St. Louis, MO) or GIBCO BRL (Gaithersburg, MD). Stock solutions of TPMPA and 3-APMPA (0.1–100 mM) were made up in H_2O.

Results

Antagonism of rat retinal $GABA_C$ receptors. The effects of TPMPA on $GABA_C$ receptors were assayed in *X. laevis* oocytes expressing cloned human ρ1 GABA receptor subunits (16, 19). The approximate potency of antagonism was first assessed by measuring the inhibition of membrane current responses activated by 0.5 μM GABA; these currents were ~7% of the maximum GABA response (Fig. 2, *top*). TPMPA induced threshold levels of inhibition at 0.1 μM; the concentration required to reduced the response by 50% (IC_{50}) was ~0.7 μM; and 10 μM TPMPA inhibited responses by 90%. As reported previously (11–19), kinetics of receptor activation and drug binding were slow compared with $GABA_A$ receptors. Applied alone, TPMPA at concentrations of ≤100 μM did not elicit a membrane current response, indicating the drug does not have agonist activity at ρ1 $GABA_C$ receptors.

Potency and mechanism of inhibition were then determined more accurately by measuring effects of fixed concentrations of TPMPA on the GABA concentration-response relationship (Fig. 3, *top*). Inhibition of ρ1 $GABA_C$ responses by TPMPA was fully surmountable. Three increasing concentrations of TPMPA caused progressive rightward transposition of the GABA concentration-response curve. The optimum EC_{50} under control conditions and the optimum slope value for simultaneous fit of the data were 1.2 (1.1–1.3) μM GABA and 2.8 (2.6–3.1) (numbers in parentheses are 95% confidence intervals adjusted to the linear scale), respectively (four to six measurements/data point). The apparent antagonist dissociation constant (K_b value) for TPMPA calculated from this fit was 2.1 (1.9–2.2) μM. Inhibition did not significantly deviate from the competitive model ($F_{3,100} = 0.70$). In confirmation of this, a conventional Schild regression of the data gave a slope of 1.000 ± 0.001 and a K_b value of 2.3 μM.

Antagonism of rat brain $GABA_A$ receptors. Inhibition of $GABA_A$ receptors was measured in oocytes expressing rat cerebral cortex poly(A)$^+$ RNA (12). This preparation gives a mixed population of $GABA_A$ receptor subtypes, presumably dominated by the most common subunits and the most favored subunit combinations. As described for ρ1 $GABA_C$ receptors, potency of inhibition was first gauged by measuring the effects of TPMPA on responses elicited by a fixed concentration of GABA (Fig. 2, *bottom*). For comparison with $GABA_C$ receptors, TPMPA was again tested on currents constituting ~7% of the maximum GABA response; in this case, responses were activated by 10 μM GABA. For $GABA_A$ receptors, 10 μM TPMPA induced only threshold levels of inhibition, the IC_{50} was ~200 μM, and 1 mM TPMPA blocked the

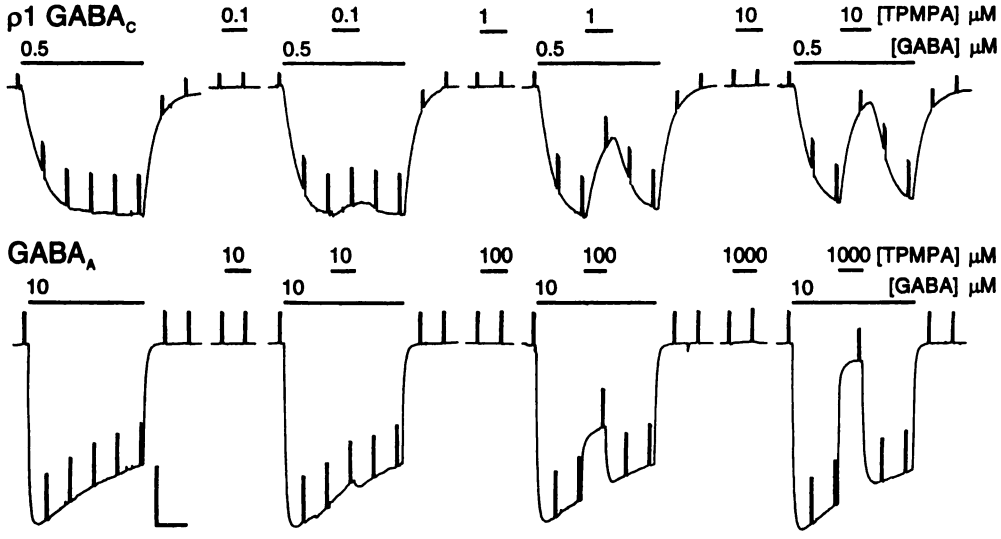

Fig. 2. Sample records comparing inhibition of $GABA_C$ and $GABA_A$ receptors by TPMPA. *Top*, inhibition of $GABA_C$ receptors in a *X. laevis* oocyte expressing cRNA encoding the human ρ1 GABA receptor subunit. *Bottom*, inhibition of $GABA_A$ receptors in an oocyte expressing rat cerebral cortex poly(A)$^+$ RNA. In each case, the series of records are taken from the same oocyte. *Bars*, drug application. The holding potential was −70 mV, with a train of brief +10-mV steps (*upward deflections*) used to help time drug applications. Capacitative transients generated on steps in voltage have been deleted from records. Individual records were each separated by 2–5-min intervals of wash (deleted). *Top scale bar*, 20 nA and 2 min; *bottom scale bar*, 50 nA and 1 min.

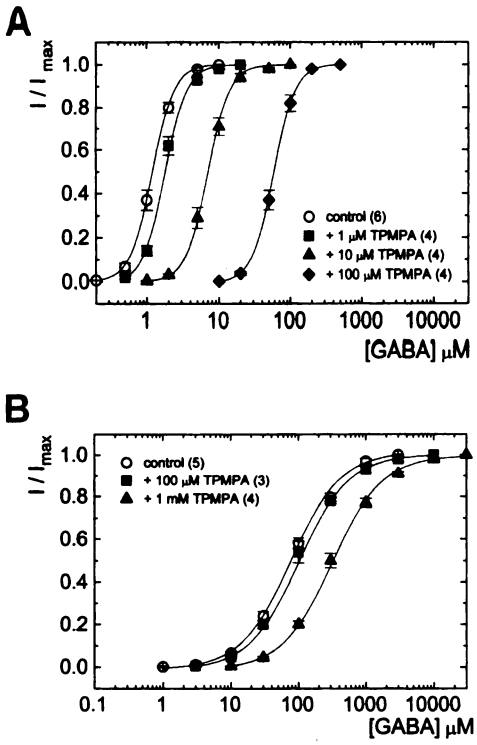

Fig. 3. Inhibition of human ρ1 GABA$_C$ receptors and rat brain GABA$_A$ receptors by TPMPA. *Top*, effect of three fixed concentrations of TPMPA on the GABA concentration-response relationship in oocytes expressing cloned rat ρ1 GABA receptor subunits. Current ranges and mean maximum response were 252 to 450 and 330 ± 50 nA, respectively (four to six measurements/data point). *Bottom*, effect of two high concentrations of TPMPA on the GABA concentration-response relationship in oocytes expressing native rat cerebral cortex poly(A)$^+$ RNA. Current ranges and mean maximum response were 350 to 800 and 570 ± 100 nA, respectively (three to five measurements/data point). Data are the mean ± standard error expressed as a fraction of the maximum GABA response. *Parentheses*, number of cells examined; *smooth curves*, best simultaneous fit of the data with equation 2. Optimum control EC$_{50}$ and slope values for the simultaneous fit are given in the text. *Top*, EC$_{50}$ and slope values for individual fits of control and 1, 10 and 100 μM TPMPA data: 1.2 μM, 2.9; 1.7 μM, 3.1; 7.1 μM, 2.6; and 60 μM, 2.9, respectively (not plotted). *Bottom*, EC$_{50}$ and slope values for individual fits of control and 100 and 1000 μM TPMPA data: 81 μM, 1.2; 93 μM, 1.2; and 330 μM, 1.1, respectively (not plotted).

current by ~90%. Applied alone, TPMPA at concentrations of ≤1 mM did not activate currents. This indicates that the drug is not a partial agonist for GABA$_A$ receptors or an agonist for any of the numerous receptors and channels coexpressed in oocytes injected with rat cerebral cortex poly(A)$^+$ RNA (32).

Potency and mechanism of inhibition were assessed by assaying the effects of TPMPA on the GABA concentration-response relationship (Fig. 3, *bottom*). Oocytes were pretreated with TPMPA for ~30 sec before receptor activation with GABA, and response amplitudes were measured at the peak of the GABA response. High concentrations of TPMPA caused modest rightward shifts in the GABA concentration-response curve. The optimum control EC$_{50}$ value and optimum slope value for simultaneous fit of the data were 78 (73–83) μM GABA and 1.2 (1.1–1.2), respectively (three to five measurements/data point). The K_b value for TPMPA calculated from this fit was 320 (280–370) μM. Inhibition did not significantly deviate from the competitive model ($F_{2,90}$ = 2.2).

Effects on rat brain GABA$_B$ receptors. Effects of TPMPA on rat brain GABA$_B$ receptors were measured with whole-cell patch recordings from pyramidal neurons in hippocampal slices (33). GABA$_A$ receptors were blocked by bathing slices in 20 μM bicuculline. Under these conditions, activation of GABA$_B$ receptors at the Schaffer collateral-CA1 synapses leads to inhibition of evoked excitatory postsynaptic currents (35).

Agonist activity on GABA$_B$ receptors was assessed by comparing the effects of TPMPA with those of the GABA$_B$ receptor agonist 3-APMPA (24). 3-APMPA induced potent reversible inhibition of evoked excitatory synaptic currents (Fig. 4). The threshold for inducing inhibition was ~0.01 μM, and 10 μM 3-APMPA induced >90% reduction in current. The IC$_{50}$ and slope values for the 3-APMPA concentration-inhibition curve were 0.23 (0.19–0.27) μM and −1.3 (−1.6 to −1.0), respectively (two to five measurements/data point) (Fig. 5). In contrast, TPMPA caused threshold inhibition between 10–50 μM and ~50% inhibition at 500 μM (Fig. 4). Concentrations of TPMPA of >500 μM were not tested. The IC$_{50}$ and slope values for the TPMPA concentration-inhibition curves were 490 (380–640) μM and −0.74 (−0.93–−0.55), respectively (two to eight measurements/data point) (Fig. 5); this curve was fit assuming full inhibition at millimolar concentrations of TPMPA.

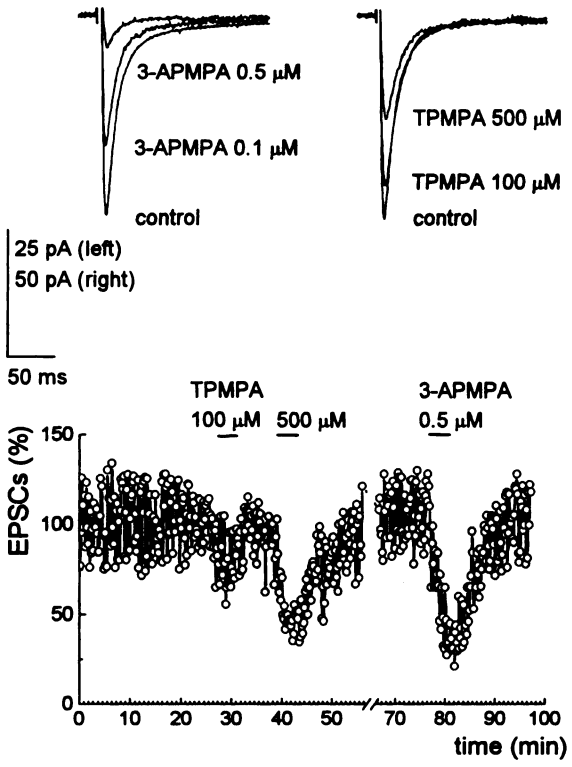

Fig. 4. Actions of TPMPA and 3-APMPA on excitatory synaptic currents in whole-cell patch-clamped pyramidal neurons: assays to detect GABA$_B$ receptor agonism. *Top*, sample records comparing reductions in amplitude of excitatory postsynaptic currents (*EPSCs*) induced by 3-APMPA and TPMPA in the same slice preparation. Each trace averages 12–18 excitatory postsynaptic currents recorded over a period of 2–3 min. The holding potential was −75 mV, and stimulus artifacts were deleted during preparation of the figure. *Bottom*, time course of an experiment comparing potencies of TPMPA and 3-APMPA for reducing EPSCs. *Points*, amplitude of a single excitatory postsynaptic current normalized with respect to the control current averaged over the first minutes of recording; *bars*, drug application.

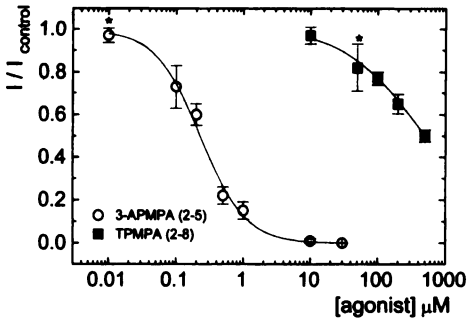

Fig. 5. Low-potency activation of $GABA_B$ responses by TPMPA. Concentration-inhibition curves comparing the relative potencies of 3-APMPA and TPMPA for inhibition of evoked excitatory synaptic currents were measured by whole-cell clamp recording from CA1 pyramidal cells in hippocampal slices. *Smooth curves*, best fits of the data with the logistic equation. IC_{50} and slope values for fits of 3-APMPA and TPMPA data are given in the text. *, Data points where only two measurements were made; these points are plotted as mean ± standard deviation.

To test whether TPMPA had any antagonist activity against $GABA_B$ receptors, we assayed for reversal of inhibition induced by 3-APMPA (Fig. 6). In these experiments, the effects of coapplied 10 or 100 μM TPMPA were measured on inhibition induced by 0.2 μM 3-APMPA. In one set of experiments, 0.2 μM 3-APMPA caused 34 ± 12% reduction in synaptic currents (mean ± standard deviation, four measurements/data point). Applied with 10 μM TPMPA, a concentration that causes minimal inhibition, the reduction was 31 ± 12%. These values are not statistically different ($p > 0.5$). In a separate set of experiments, 0.2 μM 3-APMPA applied alone caused 45 ± 15% reduction in synaptic currents (four measurements/data point). When applied with 100 μM TPMPA, a concentration that causes ~20% inhibition of current, the reduction was 44 ± 17%.

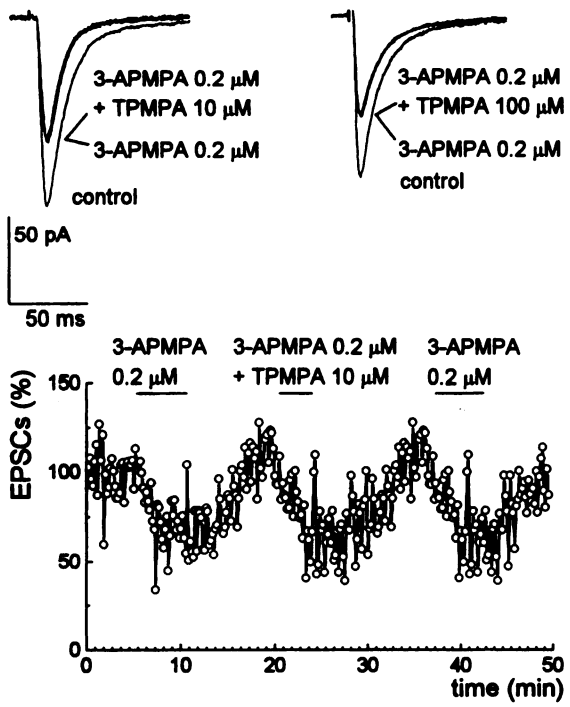

Fig. 6. Actions of TPMPA and 3-APMPA on excitatory synaptic currents in whole-cell patch-clamped pyramidal neurons: assays to detect antagonism of $GABA_B$ receptors. *Top*, sample records measuring effects of 10 and 100 μM TPMPA on reductions in amplitude of excitatory postsynaptic currents (*EPSCs*) induced by 0.2 μM 3-APMPA. Recordings were made as described in the legend to Fig. 4. *Bottom*, time course of an experiment in which the effects of TPMPA on inhibition of excitatory postsynaptic currents induced by 3-APMPA were assayed. *Points*, amplitude of a single excitatory postsynaptic current normalized with respect to the control current averaged over the first minutes of recording; *bars*, drug application.

Discussion

Subtype-selective antagonism of $GABA_C$ receptors. Our experiments indicate that TPMPA is a competitive antagonist of rat ρ1 $GABA_C$ receptors (K_b = ~2 μM) and that TPMPA has >100-fold selectivity for $GABA_C$ receptors compared with $GABA_A$ or $GABA_B$ receptors. Unlike imidazole-4-acetic acid, a compound previously reported to have selective effects on $GABA_C$ receptors (17, 18), TPMPA is not a partial agonist/antagonist.

Design of selective $GABA_C$ receptor antagonists. The pharmacology of TPMPA shows that our strategy of combining a methylphosphinic acid group with a $GABA_A$ receptor-selective ligand worked surprisingly well in generating a $GABA_C$-selective antagonist. First, the affinity of TPMPA for $GABA_C$ receptors is only slightly lower than that of 3-APMPA (15), indicating that the tetrahydropyridine ring has not greatly compromised affinity. Second, TPMPA shows only weak antagonism of $GABA_A$ receptors, indicating that the methylphosphinic acid group retains the property of prohibiting binding. Last, and most important, TPMPA has very weak agonist activity at $GABA_B$ receptors, indicating that the tetrahydropyridine ring is sufficient to reduce potency >1000-fold with respect to 3-APMPA.

Our results strongly suggest that the strategy of coupling substituted phosphinic acid groups to other types of GABA analogue will be a viable approach for designing additional $GABA_C$-selective antagonists (15). We suggest that good candidates are the methylphosphinic acid analogues of imidazole-4-acetic acid and *Z*-3-(amidinothio)propenonic acid (15, 17). Separate experiments indicate that 1,2,5,6-tetrahydropyridine-4-ethylphosphinic acid, the ethyl analogue of TPMPA, is also a $GABA_C$ receptor antagonist,[1] whereas CGP 35348, the diethoxymethyl analogue of 3-APMPA, is inactive (15). These results suggest that there are distinct size limitations for other possible phosphinic acid substituents. The search for additional $GABA_C$-selective antagonists remains important because only ligands with submicromolar affinities are likely to make promising candidates as subtype-selective radioligands.

Potency and selectivity *in situ*. TPMPA is a competitive antagonist of ρ1 $GABA_C$ receptors and, at a much lower potency, of $GABA_A$ receptors. $GABA_C$ receptors are characterized by high affinity for GABA (EC_{50} ≃ 1 μM), strong cooperativity (Hill slope ≃ 3), slow activation and deactivation kinetics, and little or no desensitization (11, 16, 19, 20). In the outer retina, signaling between neurons is often in the form of graded potentials, as opposed to fast synaptic events. It is therefore tempting to speculate that $GABA_C$ receptors are important for the tonic regulation of excitability. If this is the case, then one can envisage TPMPA competing under approximately steady state conditions against low micromo-

[1] L. E. Overman and R. Miledi, unpublished observations.

lar or submicromolar concentrations of GABA. Activity of the compound under these conditions *in situ* should correspond to that measured in oocyte assays with nonsaturating concentrations of agonist. If, on the other hand, $GABA_C$ receptors are exposed to sustained high concentrations of GABA, then the high affinity of the receptors for agonist will render TPMPA disappointingly weak when assayed *in vivo*, or in retinal slice or eye cup preparations.

Inhibition of $GABA_A$ receptors will seldom be under steady state conditions. Gating of $GABA_A$ receptors is associated with fast inhibitory postsynaptic currents activated by vesicular release of GABA (e.g., 36). Potency of TPMPA for inhibiting $GABA_A$ receptor current *in situ* will therefore depend on agonist and antagonist binding kinetics. In our experiments, oocytes expressing $GABA_A$ receptors were pretreated with TPMPA before receptor activation and inhibition was measured on the peak GABA response. Potency measured under these conditions should approximate potency measured at the synaptic level. However, drug application to oocytes is too slow to resolve the more rapid components of $GABA_A$ receptor activation and desensitization (36, 37), so our experiments may somewhat underestimate antagonist potency *in situ*.

For $GABA_B$ receptors, the potency of TPMPA agonism was measured in a slice preparation and should closely approximate the true *in situ* potency. The uncertainty here is which subtype or subtypes of $GABA_B$ receptors are being assayed. Previous studies indicate that reduction in excitatory synaptic currents is primarily mediated by activation of presynaptic $GABA_B$ receptors (e.g., 24, 38, and 39). In the current study, however, we could not rule out the possibility that postsynaptic $GABA_B$ receptors contribute to the effect. The important point is that TPMPA is a weak ligand for whatever $GABA_B$ receptors are involved in the reduction of excitatory synaptic currents, whether they are presynaptic or postsynaptic. The concentration-inhibition curve for TPMPA, extrapolating full inhibition, has a low slope compared with 3-APMPA: −0.74 and −1.3, respectively. This raises the possibility that TPMPA is a weak partial agonist at $GABA_B$ receptors. We did not assay high concentrations of TPMPA against inhibition induced by saturating concentrations of APMPA to test for the predicted reversal of inhibition. It should also be noted that though the hippocampal slice preparation is a robust assay of $GABA_B$ receptor activity (35), it is only an indirect assay. It remains possible that high concentrations of TPMPA cause inhibition of excitatory postsynaptic currents by a mechanism that does not involve direct activation of $GABA_B$ receptors (i.e., that we are overstating the potency of TPMPA for $GABA_B$ receptors).

Further pharmacological profiling of TPMPA. Our results indicate that TPMPA has good levels of selectivity for ρ1 $GABA_C$ receptors compared with $GABA_A$ and $GABA_B$ receptors. Some additional pharmacological profiling would be desirable before the compound can be used with real confidence to probe $GABA_C$ receptor function. In particular, it will be reassuring to test TPMPA on ρ2 $GABA_C$ receptors (18), on distinct and defined subtypes of $GABA_B$ receptors (2, 35), and as a substrate/inhibitor for the various subtypes of GABA transporters (40). Needless to say, it will also be necessary to assay TPMPA against other types of mammalian $GABA_C$ receptors as these are identified, characterized, and eventually cloned; examples include a GABA autoreceptor with atypical pharmacology described in rat spinal cord (41), GABA receptors transiently expressed in postnatal rat hippocampus (42), and binding sites with $GABA_C$-like properties in rat cerebellum (43).

Some of the concerns about TPMPA specificity can be addressed indirectly, albeit with a degree of uncertainty. For example, the pharmacology of ρ2 $GABA_C$ receptors is generally similar to that of ρ1 receptors (17, 18), so we do not anticipate that TPMPA will show any great difference in potency between the two subtypes. With respect to GABA transporters, isoguvacine is not a substrate/inhibitor of transport in cultured mouse astrocytes, rat brain slices, or synaptosomes (44). This implies that TPMPA, an analogue of isoguvacine, will be similarly inactive in GABA transport.

Finally, certain substituted phosphinic acid analogues of GABA have been shown to be systemically active $GABA_B$ receptor ligands in rodents on subcutaneous or oral administration (45). If TPMPA is systemically active, and therefore amenable to behavioral testing, it will be important to screen the compound against a broad panel of receptors, enzymes, and channels to test for activity outside GABAergic systems.

Conclusion. TPMPA is a water-soluble GABA receptor antagonist that shows strong selectivity for $GABA_C$ receptors composed of ρ1 subunits. Our results suggest that TPMPA will be a useful pharmacological tool with which to investigate the function of ρ-containing $GABA_C$ receptors in mammalian retina and in any other parts of the nervous system in which these receptors are present.

References

1. MacDonald, R. L., and R. W. Olsen. $GABA_A$ receptor channels. *Annu. Rev. Neurosci.* **17**:569–602 (1994).
2. Kerr, D. I., and J. Ong. $GABA_B$ receptors. *Pharmacol. Ther.* **67**:187–246 (1995).
3. Johnston, G. A. R. $GABA_C$ receptors: molecular biology, pharmacology and physiology, in *The GABA Receptors* (S. J. Enna and N. G. Bowery, eds.). in press (1996).
4. Johnston, G. A. R. Multiplicity of GABA receptors, in *Benzodiazepine/GABA Receptors and Chloride Channels: Structural and Functional Properties* (R. W. Olsen and J. C. Venter, eds.). Allan R. Liss, New York, 57–71 (1986).
5. Bormann, J., and A. Feigenspan. $GABA_C$ receptors. *Trends Neurosci.* **18**:515–519 (1995).
6. Cutting, G. R., L. Lu, B. F. O'Hara, L. M. Kasch, C. Montrose-Rafizdeh, D. M. Donovan, S. Shimada, S. E. Antonarakis, W. B. Guggino, G. R. Uhl, and H. H. Kazazian. Cloning of the γ-aminobutyric acid (GABA) ρ1 cDNA: a GABA receptor subunit highly expressed in the retina. *Proc. Natl. Acad. Sci. USA* **88**:2673–2677 (1991).
7. Wang, T.-L., W. B. Guggino, and G. R. Cutting. A novel γ-aminobutyric acid receptor subunit (ρ2) cloned from human retina forms bicuculline-insensitive homooligomeric receptors in *Xenopus* oocytes. *J. Neurosci.* **14**: 6524–6531 (1994).
8. Enz, R., J. H. Brandstatter, E. Hartveit, H. Wassle, and J. Bormann. Expression of GABA receptor ρ1 and ρ2 subunits in the retina and brain of the rat. *Eur. J. Neurosci.* **7**:1495–1501 (1995).
9. Calvo, D. J., and R. Miledi. Activation of GABA rho1 receptors by glycine and *beta*-alanine. *Neuroreports* **6**:1118–20 (1995).
10. Darlinson, M. G., and B. E. Albrecht. $GABA_A$ receptor subtypes: which, where and why? *Semin. Neurosci.* **7**:115–126 (1995).
11. Polenzani, L., R. M. Woodward, and R. Miledi. Expression of mammalian γ-aminobutyric acid receptors with distinct pharmacology in *Xenopus* oocytes. *Proc. Natl. Acad. Sci. USA* **88**:4318–4322 (1991).
12. Woodward, R. M., L. Polenzani, and R. Miledi. Effects of steroids on γ-aminobutyric acid receptors expressed in *Xenopus* oocytes by poly(A)$^+$ RNA from mammalian brain and retina. *Mol. Pharmacol.* **41**:89–103 (1992).
13. Woodward, R. M., L. Polenzani, and R. Miledi. Effects of hexachlorocyclohexanes on γ-aminobutyric acid receptors expressed in *Xenopus* oocytes by poly(A)$^+$ RNA from mammalian brain and retina. *Mol. Pharmacol.* **41**: 1107–1115 (1992).
14. Woodward, R. M., L. Polenzani, and R. Miledi. Characterization of bicuculline/baclofen-insensitive γ-aminobutyric acid receptors expressed in *Xenopus* oocytes. I. Effects of Cl^- channel inhibitors. *Mol. Pharmacol.* **42**: 165–173 (1992).

15. Woodward, R. M., L. Polenzani, and R. Miledi. Characterization of bicuculline/baclofen-insensitive γ-aminobutyric acid receptors expressed in *Xenopus* oocytes. II. Pharmacology of γ-aminobutyric $acid_A$ and γ-aminobutyric $acid_B$ receptor agonists and antagonists. *Mol. Pharmacol.* **43**:609–625 (1993).
16. Shimada, S., G. R. Cutting, and G. R. Uhl. γ-Aminobutyric acid A or C receptor? γ-Aminobutyric acid ρ1 receptor RNA induces bicuculline-, barbiturate-, and benzodiazepine-insensitive γ-aminobutyric acid responses in *Xenopus* oocytes. *Mol. Pharmacol.* **41**:683–687 (1992).
17. Kusama, T., C. E. Spivak, P. Whiting, V. L. Dawson, J. C. Schaeffer, and G. R. Uhl. Pharmacology of GABA ρ1 and GABA α/β receptors expressed in *Xenopus* oocytes and COS cells. *Br. J. Pharmacol.* **109**:200–206 (1993).
18. Kusama, T., T. L. Wang, W. B. Guggino, G. R Cutting, and G. R. Uhl. GABA ρ2 receptor pharmacological profile: GABA recognition similarities to ρ1. *Eur. J. Pharmacol.* **245**:83–84 (1993).
19. Calvo, D. J., A. E. Vasquez, and R. Miledi. Cationic modulation of ρ1-type γ-aminobutyrate receptors expressed in *Xenopus* oocytes. *Proc. Natl. Acad. Sci. USA* **91**:12725–12729 (1994).
20. Feigenspan, A., H. Wassle, and J. Bormann. Pharmacology of GABA receptor Cl^- channels in rat retinal bipolar cells. *Nature (Lond.)* **361**:159–162 (1993).
21. Feigenspan, A., and J. Bormann. Differential pharmacology of $GABA_A$ and $GABA_C$ receptors on rat retinal bipolar cells. *Eur. J. Pharmacol.* **288**:97–104 (1994).
22. Qian, H., and J. E. Dowling. Novel GABA responses from rod-driven retinal horizontal cells. *Nature (Lond.)* **361**:162–164 (1993).
23. Djamgoz, M. B. A. Diversity of GABA receptors in the vertebrate outer retina. *Trends Neurosci.* **18**:118–120 (1995).
24. Seabrook, G. R., W. Howson, and M. G. Lacey. Electrophysiological characterization of potent agonists and antagonists at pre- and postsynaptic $GABA_B$ receptors on neurons in brain slices. *Br. J. Pharmacol.* **101**:949–957 (1990).
25. Pan, Z.-H., and S. A. Lipton. Multiple GABA receptor subtypes mediate inhibition of calcium influx at rat retinal bipolar terminals. *J. Neurosci.* **15**:2668–2679 (1995).
26. Hill, D. R., and N. G. Bowery. [^{3}H]-Baclofen and [^{3}H]-GABA bind to bicuculline-insensitive $GABA_B$ sites in rat brain. *Nature (Lond.)* **290**:149–152 (1981).
27. Arnold, H., L. E. Overman, M. J. Sharp, and M. C. Witschel. (E)-1-Benzyl-3-(1-iodoethylidene)piperidine: nucleophile-promoted alkyne-iminium ion cyclizations. *Org. Synth.* **70**:111–119 (1991).
28. Petrov, K. A., N. K. Bliznyuk, Y. N. Studnev, and A. F. Kolomiets. Monoalkoxymethylthiophosphates and monoalkoxymethylphosphonites. *J. Gen. Chem. USSR* **31**:168–171 (1961).
29. Hirao, T., T. Masunaga, Y. Ohshiro, and T. Agawa. Stereoselective synthesis of vinylphosphonate. *Tetrahedron Lett.* **21**:3595–3598 (1980).
30. Kozyukov, V. P., V. D. Sheludyakov, and V. F. Miromov. Synthesis of carbofunctionalized organic silicon compounds containing isocyanate and chloroformate groups. *J. Gen. Chem. USSR* **38**:1133–1138 (1968).
31. Hawkinson, J. E., J. A. Drewe, C. L. Kimbrough, J.-S. Chen, D. J. Hogenkamp, N. C. Lan, K. W. Gee, K.-Z. Shen, E. R. Whittemore, and R. M. Woodward. 3α-Hydroxy-3β-trifluoromethyl-5α-pregnan-20-one (Co 2–1970): a partial agonist at the neuroactive steroid site of the γ-aminobutyric $acid_A$ receptor. *Mol. Pharmacol.* **49**:897–906 (1996).
32. Miledi, R., I. Parker, and K. Sumikawa. Transplanting receptors from brains into oocytes, in *Fidia Research Foundation Neuroscience Award Lectures*. Raven Press, New York, 57–90 (1989).
33. Edwards, F. A., A. Konnerth, B. Sakmann, and T. Takahashi. A thin slice preparation for patch-clamp recordings from neurones of the mammalian central nervous system. *Pflueg. Arch. Eur. J. Physiol.* **414**:600–612 (1989).
34. Woodward, R. M., J. E. Huettner, J. Guastella, J. F. W. Keana, and E. Weber. *In vitro* pharmacology of ACEA-1021 and ACEA-1031: systemically active quinoxalinediones with high affinity and selectivity for *N*-methyl-D-aspartate receptor glycine sites. *Mol. Pharmacol.* **47**:568–581 (1995).
35. Dutar, P., and R. A. Nicoll. Pre- and postsynaptic $GABA_B$ receptors in the hippocampus have different pharmacological properties. *Neuron* **1**:585–591 (1988).
36. Edwards, F. A., A. Konnerth, and B. Sakmann. Quantal analysis of inhibitory synaptic transmission in the dentate gyrus of rat hippocampal slices: a patch clamp study. *J. Physiol. (Lond.)* **430**:213–249 (1990).
37. Jones, M. V., and G. L. Westbrook. Desensitized states prolong $GABA_A$ channel responses to brief agonist pulses. *Neuron* **15**:181–191 (1995).
38. Thompson, S. M., and B. H. Gahwiler. Comparison of the actions of baclofen at pre- and post-synaptic receptors in the rat hippocampus *in vitro*. *J. Physiol. (Lond.)* **451**:329–345 (1992).
39. Pfrieger, F. W., K. Gottmann, and H. D. Lux. Kinetics of $GABA_B$ receptor-mediated inhibition of calcium currents and excitatory synaptic transmission in hippocampal neurons *in vitro*. *Neuron* **12**:97–107 (1994).
40. Amara, S. G., and M. J. Kuhar. Neurotransmitter transporters: recent progress. *Annu. Rev. Neurosci.* **16**:73–93 (1993).
41. Raiteri, M., G. Pellegrini, C. Cantoni, and G. Bonanno. A novel type of GABA receptor in rat spinal cord? *Arch. Pharmacol.* **340**:666–670 (1989).
42. Martina, M., F. Strata, and E. Cherubini. Whole cell and single channel properties of a new GABA receptor transiently expressed in the hippocampus. *J. Neurophysiol.* **73**:902–906 (1995).
43. Drew, C. A., and G. A. R. Johnston. Bicuculline- and baclofen-insensitive γ-aminobutyric acid binding to rat cerebellar membranes. *J. Neurochem.* **58**:1087–1092 (1992).
44. Krogsgaard-Larsen, P. Inhibitors of the GABA uptake systems. *Mol. Cell. Biochem.* **31**:105–121 (1980).
45. Froestl, W., S. J. Mickel, R. G. Hall., G. von Sprecher, D. Strub, P. A. Baumann, F. Brugger, C. Gentsch, J. Jaekel, H.-R. Olpe, G. Rihs, A. Vassout, P. C. Waldmeier, and H. Bittiger. Phosphinic acid analogues of GABA. 1. New potent and selective $GABA_B$ agonists. *J. Med. Chem.* **38**:3297–3312 (1995).
46. Murata, Y., R. M. Woodward, R. Miledi, and L. E. Overman. The first selective agonist for a $GABA_C$ receptor. *Biomed. Chem. Lett.* **6**:2073–2076 (1996).

Send reprint requests to: Dr. R. Miledi, Department of Psychobiology, University of California, Irvine, CA 92697-4550. E-mail: rmiledi@uci.edu