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Steroid Hormone Metabolites Are Barbiturate-Like Modulators of the GABA Receptor

MARIA DOROTA MAJEWSKA,* NEIL L. HARRISON, ROCHELLE D. SCHWARTZ, JEFFERY L. BARKER, STEVEN M. PAUL

Two metabolites of the steroid hormones progesterone and deoxycorticosterone, 3α-hydroxy-5α-dihydroprogesterone and 3α,5α-tetrahydrodeoxycorticosterone, are potent barbiturate-like ligands of the γ-aminobutyric acid (GABA) receptor–chloride ion channel complex. At concentrations between 10^{-7} and 10^{-5} *M* both steroids inhibited binding of the convulsant *t*-butylbicyclophosphorothionate to the GABA-receptor complex and increased the binding of the benzodiazepine flunitrazepam; they also stimulated chloride uptake (as measured by uptake of $^{36}Cl^-$) into isolated brain vesicles, and potentiated the inhibitory actions of GABA in cultured rat hippocampal and spinal cord neurons. These data may explain the ability of certain steroid hormones to rapidly alter neuronal excitability and may provide a mechanism for the anesthetic and hypnotic actions of naturally occurring and synthetic anesthetic steroids.

STEROID HORMONES ACT ON THE central nervous system (CNS) to produce diverse neuroendocrine and behavioral effects (*1*). Both adrenal and gonadal steroids interact with intracellular receptors in the CNS and trigger genomically directed alterations in protein synthesis, which occur in minutes to hours (*2*). In addition, many steroids produce more rapid alterations in CNS excitability (*1*). Over 40 years ago Selye (*3*) described the rapid and reversible CNS depressant actions of various steroids in the rat. The gonadal steroid progesterone, and the mineralocorticoid deoxycorticosterone, as well as several of their metabolites, were the most potent among a series of steroids in inducing sedation and anesthesia (*4*). On the basis of these observations a class of steroidal anesthetics was developed and has been used clinically (*5*).

The mechanisms responsible for the rapid effects of steroids on neuronal excitability are poorly understood, although the short latency (seconds to minutes) of the effects makes it unlikely that they are mediated by "classical" intracellular receptors. The anesthetic and hypnotic actions of certain drugs, including the benzodiazepines (*6*), barbiturates (*7*), and the anesthetic steroid 3α-hydroxy-5α-pregnane-11,20-dione (alphaxalone) (*8*) may be due in part to their enhancement of the inhibitory action of the neurotransmitter γ-aminobutyric acid (GABA). The potentiation of GABA-mediated synaptic inhibition by these drugs occurs by a direct interaction with $GABA_A$ receptors, which are coupled to chloride (Cl^-) ion channels (*6–9*). The $GABA_A$ receptor is an oligomeric receptor complex consisting of several subunits with independent but interacting binding sites for GABA, benzodiazepines, and barbiturates (*9*). Because the $GABA_A$-receptor complex is an important site of anesthetic and hypnotic drug action, we examined a series of naturally occurring steroids for their ability to interact with one or more sites on this receptor complex. We now report that the ring A reduced metabolites of progesterone and deoxycorticosterone—namely, 3α-hydroxy-5α-dihydroprogesterone (3α-OH-DHP) and 3α,5α-tetrahydrodeoxycorticosterone (3α-THDOC), respectively—are potent modulators of the GABA-receptor complex and interact at a site close to or identical with that for barbiturates.

A series of steroids was tested for their ability to inhibit the specific binding of the ^{35}S-labeled convulsant *t*-butylbicyclophosphorothionate ([^{35}S]TBPS), a ligand that labels a site close to or on the GABA-operated Cl^- channel (*10*). The specific binding of [^{35}S]TBPS to brain membranes is inhibited both by barbiturates and by GABA antagonists such as picrotoxin, and there is a good correlation between the pharmacological potencies of these compounds and their ability to displace [^{35}S]TBPS binding (*10*). Both 3α-OH-DHP and 3α-THDOC were relatively potent inhibitors of [^{35}S]TBPS binding to the $GABA_A$ receptor–Cl^- channel complex in crude synaptosomal membranes from rat

M. D. Majewska, R. D. Schwartz, S. M. Paul, Sections on Molecular Pharmacology and Preclinical Studies, Clinical Neuroscience Branch, National Institute of Mental Health, Bethesda, MD 20892.
N. L. Harrison and J. L. Barker, Laboratory of Neurophysiology, National Institute of Neurological and Communicative Disorders and Stroke, National Institutes of Health, Bethesda, MD 20892.

*To whom correspondence should be addressed.

brain; a variety of other chemically related steroids (*11*), including the parent compounds 5α-dihydroprogesterone and 5α-dihydrodeoxycorticosterone, were inactive or weakly active (Fig. 1A). 3α-OH-DHP and 3α-THDOC were 700 to 1000 times more potent than the prototypic anesthetic barbiturate pentobarbital in displacing [^{35}S]-TBPS. A Scatchard plot of the binding data indicated that both steroids decreased the apparent maximal binding capacity (B_{max}) and also decreased the apparent affinity or dissociation constant (K_d) of [^{35}S]TBPS binding (Fig. 1B). This uncompetitive inhibition of [^{35}S]TBPS binding by 3α-OH-DHP and 3α-THDOC was similar to that seen with the anesthetic barbiturates. These steroids (like barbiturates) probably act at sites on the GABA-receptor complex that are not identical with those that bind [^{35}S]TBPS (*12*).

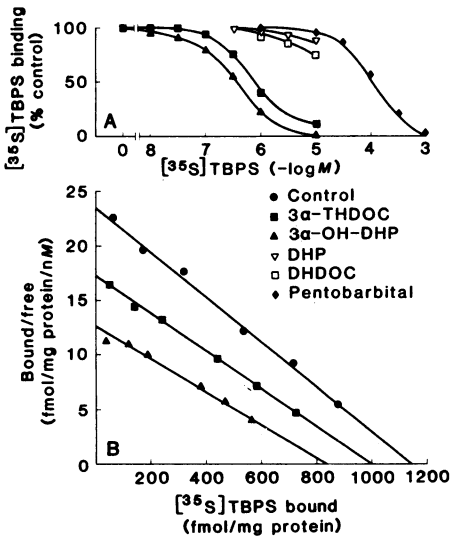

Fig. 1. (A) The effects of various steroids on the specific binding of [^{35}S]TBPS to crude synaptosomes. The synaptosomal membrane preparation (*25*) (approximately 250 μg of protein per assay) was incubated (37°C) for 5 minutes with the various steroids or pentobarbital. [^{35}S]TBPS (final concentration of 5 n*M*; 92 Ci/mmol, NEN, Boston, MA) was then added to a total volume of 0.5 ml. After a 60-minute incubation at 37°C, the reaction was terminated by rapid filtration over Whatman GF-B glass fiber filters with a Brandel Tissue Harvester (Gaithersburg, MD). The filters were washed once with 4 ml of 50 m*M* tris-HCl containing 50 m*M* NaCl (*p*H 7.4). All steroids were initially dissolved in 48% ethanol at a concentration of 1 m*M* and were diluted in Krebs-phosphate buffer to the desired concentration. Radioactivity retained on the filters was measured by conventional liquid scintillation spectrometry. Protein was determined by the Bio-Rad protein method (Bio-Rad, Richmond, CA) with bovine serum albumin as standard. The specific binding of [^{35}S]TBPS was calculated from the difference between total and nonspecific binding; the latter was determined by the addition of picrotoxin (200 μ*M*) to a parallel set of tubes. At a ligand concentration of 5 n*M*, specific [^{35}S]TBPS binding was approximately 70% of the total binding. Data are from a single experiment carried out in triplicate and are representative of three such experiments which yielded similar results. Control incubations contained ethanol alone at the corresponding concentrations required to keep the steroids in solution. The latter resulted in no demonstrable inhibition of [^{35}S]TBPS binding until the ethanol concentration was 0.48% (corresponding to a steroid concentration of 10 μ*M*), which resulted in a 15 to 20% inhibition of [^{35}S]TBPS binding. (B) Scatchard analysis of the effects of 3α-OH-DHP (300 n*M*) and 3α-THDOC (300 n*M*) on [^{35}S]TBPS binding to the crude synaptosomal fraction of rat brain. Synaptosomal membranes were prepared and incubated with steroid for 5 minutes as described in (A). [^{35}S]TBPS (2 to 200 n*M*) was added and incubated as described in (A). Values for the B_{max} and apparent K_d were determined and analyzed with computer-assisted linear regression analysis (*26*).

Because barbiturates stimulate the specific binding of benzodiazepines in vitro in proportion to their CNS depressant potencies (*13*), we examined the effects of 3α-OH-DHP and 3α-THDOC on the binding of the benzodiazepine flunitrazepam to synaptosomes from rat forebrain. Both steroids stimulated the specific binding of [^{3}H]flunitrazepam to the benzodiazepine receptor (Fig. 2A) and, as in the [^{35}S]TBPS binding studies, the potencies of these steroids were approximately 1000 times that of pentobarbital. Scatchard analysis of the [^{3}H]flunitrazepam binding data revealed that, like pentobarbital, both steroids increased the apparent affinity of [^{3}H]flunitrazepam binding without altering the apparent B_{max} (Fig. 2B).

These binding experiments demonstrated that 3α-OH-DHP and 3α-THDOC interact with the GABA-receptor complex in a manner similar to the barbiturates. To test if either steroid has functional activity similar to the anesthetic barbiturates or picrotoxin-like convulsants, we measured the Cl^- flux mediated by the GABA-barbiturate receptor in vitro (*14*). Barbiturates and GABA agonists increased radioactive Cl^- ($^{36}Cl^-$) uptake or efflux in a picrotoxin-sensitive fashion (*14*). 3α-OH-DHP also stimulated the uptake of $^{36}Cl^-$ into brain vesicles at concentrations as low as 300 n*M*, maximal stimulation occurring at approximately 1 μ*M* (Fig. 3); similar results were observed with 3α-THDOC. Furthermore, the stimulation of $^{36}Cl^-$ uptake by 3α-OH-DHP and 3α-THDOC, like that produced by muscimol and pentobarbital (Fig. 3, inset), was blocked by prior incubation of the vesicles with the noncompetitive GABA antagonist, picrotoxin (*15*).

In electrophysiological experiments barbiturates and alphaxalone potentiate GABA-activated Cl^- conductance at low (sedative-hypnotic) concentrations; at high (anesthetic) concentrations they stimulate Cl^- conductance directly (*7, 8, 16*). We investigated the modulation by 3α-OH-DHP and 3α-THDOC of GABA-activated Cl^- conductance in electrophysiological recordings from cultured rat hippocampal and spinal cord neurons. Under current-clamp recording conditions, application of GABA to hippocampal neurons at the resting membrane potential (−55 mV) typically resulted in a 2- to 3-mV hyperpolarization of the membrane associated with an increase in membrane conductance (Fig. 4A$_1$). Action potentials were evoked in one such neuron by depolarizing current pulses (100 msec, 0.6 nA) and, at regular intervals, the depo-

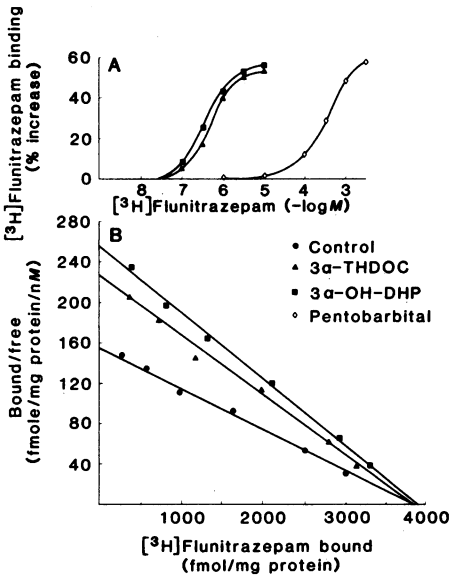

Fig. 2. (A) The effects of 3α-THDOC, 3α-OH-DHP, and pentobarbital on [^{3}H]flunitrazepam binding. The crude synaptosomal fraction was prepared as described in Fig. 1A and incubated for 5 minutes with various concentrations of steroids or pentobarbital. The binding reaction was initiated by the addition of [^{3}H]flunitrazepam (3 n*M* final concentration, 93 Ci/mmol, NEN, Boston, MA). After 20 minutes at 37°C, the reaction was terminated by rapid filtration and single wash and the radioactivity was measured as in Fig. 1. Nonspecific binding was determined in the presence of 50 μ*M* diazepam; specific binding was approximately 80% of total binding at a ligand concentration of 3 n*M*. The data are from a representative experiment carried out in triplicate and repeated four times with similar results. (B) Scatchard analysis of [^{3}H]flunitrazepam binding to synaptosomes carried out in the presence or absence of 3α-THDOC (3 μ*M*) or 3α-OH-DHP (3 μ*M*). [^{3}H]Flunitrazepam binding (2 to 100 n*M*) to crude synaptosomal membranes was carried out as in (A). Values for the B_{max} and apparent K_d were determined by computer assisted linear regression analysis (*27*). Data are from a representative experiment carried out in triplicate and repeated three times with similar results. The diluted ethanol vehicle had no effect on specific [^{3}H]flunitrazepam binding. In related experiments we observed that the stimulatory effects of 3α-THDOC and 3α-OH-DHP on [^{3}H]flunitrazepam binding were maximal when incubations were carried out at 37°C; both steroids were far less potent when binding was carried out at 0 to 4°C.

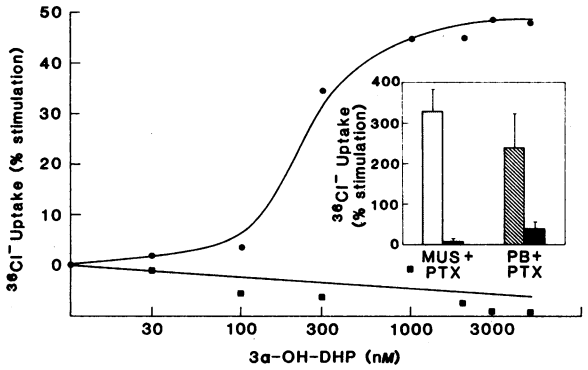

Fig. 3. The effect of 3α-OH-DHP on $^{36}Cl^-$ uptake into synaptoneurosomes. $^{36}Cl^-$ uptake was assayed as described (*14*). Synaptoneurosomes (*28*) were incubated with 3α-OH-DHP (3 μ*M*) for 20 minutes at 30°C. Picrotoxin was added 5 minutes before to the steroid. To initiate uptake, 0.5 μCi of $^{36}Cl^-$ (12.5 Ci/g, NEN) was then added to each tube, and 5 seconds later the assay mixture was diluted with 5 ml of cold buffer and then filtered over Whatman GFC glass fiber filters under vacuum. The filters were washed twice with 5 ml of ice-cold buffer and the radioactivity was determined by liquid scintillation spectrometry. Data are the mean of quadruplicate determinations at each concentration for a representative experiment. The experiment was repeated three times with similar results (*29*). (●) 3α-OH-DHP; (■) 3α-OH-DHP plus picrotoxin (200 μ*M*). (Inset) Synaptoneurosomes were incubated with picrotoxin for 5 minutes at 30°C, and then $^{36}Cl^-$ and muscimol (20 μ*M*) or pentobarbital (500 μ*M*) were added simultaneously. $^{36}Cl^-$ uptake was terminated 5 seconds later as described above. Values are the mean ± SEM of quadruplicate determinations from one representative experiment. Two other experiments yielded similar results. Stippled bar, muscimol (20 μ*M*); hatched bar, pentobarbital (500 μ*M*); solid bar, muscimol or pentobarbital plus picrotoxin (200 μ*M*).

larizing stimulus was immediately preceded by a 50-msec application of GABA (50 μ*M*) (Fig. $4A_2$). The increase in conductance produced by GABA (Fig. $4A_1$) "shunted" the membrane, so that the depolarizing current evoked only a subthreshold electrotonic potential. This "inhibitory pause" in action potential generation induced by GABA lasted for only one excitatory stimulus (Fig. $4A_2$, arrow). Application to the neuron of 3α-OH-DHP (300 n*M*) markedly increased the duration of this pause (Fig. $4A_2$). Action potential generation was unaffected during the period of application of 3α-OH-DHP alone.

In hippocampal neurons monitored with a CsCl-filled microelectrode and held at −60 mV under voltage-clamp, brief application of 1 μ*M* 3α-OH-DHP significantly increased both the peak amplitude (Fig. $4B_1$) and duration (Fig. $4B_2$) of the inward Cl^- current induced by GABA; this effect of the steroid reversed within a few minutes. A slow, inward-current response to the steroid was apparent (Fig. $4B_1$). This current was also seen in the absence of applied GABA, and resembles the direct responses elicited in cultured neurons by pentobarbital (*16*) and alphaxalone (*8*). Similar results were obtained with 3α-THDOC in hippocampal neurons and with both 3α-OH-DHP and 3α-THDOC in cultured spinal cord neurons. In contrast, 3α-OH-DHP did not potentiate glycine-activated Cl^- conductance in spinal cord neurons in our experiments.

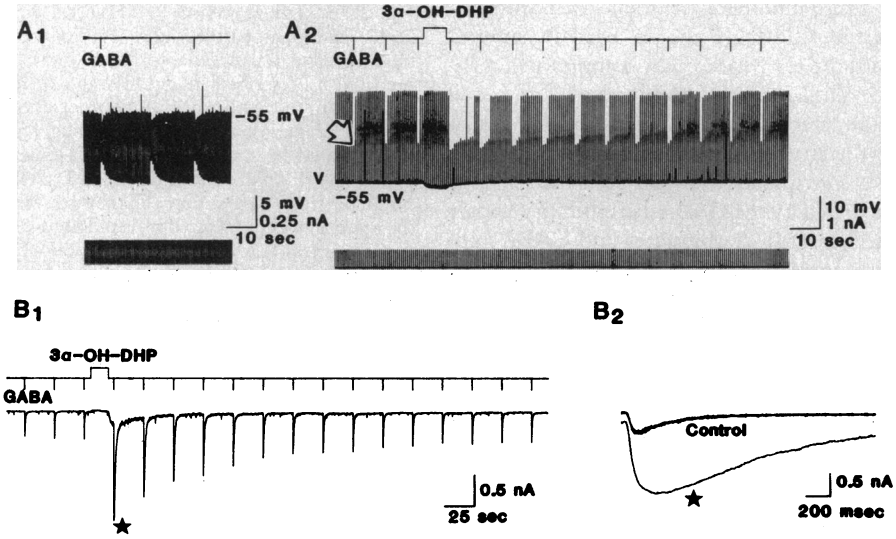

Fig. 4. The effects of 3α-OH-DHP on the inhibitory actions of GABA in recordings from cultured rat hippocampal neurons (*30*). (A_1) The effect of GABA on membrane conductance. GABA was applied in 200-msec pulses by positive pressure (<0.3 kpa) from a micropipette (tip diameter 1 to 2 μm) containing 50 μ*M* GABA placed within 2 to 3 μm of the cell soma. (A_2) The effect of GABA on action potential generation (see arrow). This inhibitory action of GABA is prolonged by 3α-OH-DHP. The steroid was applied to the cell body of the neuron from another pressure pipette. (B_1) The effect of 3α-OH-DHP on the amplitude of GABA-evoked inward Cl^- currents in a cell monitored with a 145 m*M* CsCl-filled microelectrode (*31*). (B_2) The effect of 3α-OH-DHP on the duration of GABA-evoked currents. Five superimposed current responses to GABA (2-second sweep) are shown (Control) together with the current response that immediately followed steroid application (★). In the controls, the time taken for the GABA current response to decay to half its peak value is close to 200 msec; for the response potentiated by the steroid, this half-decay time is approximately four times as long.

These data demonstrated that the naturally occurring steroids 3α-OH-DHP and 3α-THDOC are relatively potent barbiturate-like ligands of the GABA-barbiturate receptor complex. At 10^{-7} to 10^{-5}*M* both steroids (i) inhibit [^{35}S]TBPS binding to the GABA-receptor complex, (ii) increase specific [^{3}H]flunitrazepam binding, (iii) stimulate $^{36}Cl^-$ uptake in a picrotoxin-sensitive fashion into isolated brain vesicles, and (iv) potentiate the actions of GABA in activating Cl^- conductance in cultured rat hippocampal and spinal cord neurons.

3α-OH-DHP and 3α-THDOC have neurochemical and electrophysiological properties similar to those of the anesthetics pentobarbital and alphaxalone (*7, 8, 13, 16*). Therefore, the anesthetic and hypnotic properties of progesterone and deoxycorticosterone may be mediated by their metabolites, 3α-OH-DHP and 3α-THDOC, respectively. The interval between administration of progesterone and deoxycorticosterone and the onset of behavioral depression (*17*) suggests the formation of active metabolites. For example, after administration of progesterone and deoxycorticosterone to rats, the time course of CNS depression more closely parallels the formation and presence in brain of their metabolites, including 3α-OH-DHP and 3α-THDOC, than it does the brain concentration of the parent steroids (*17*).

The physiological significance of these findings is unknown. However, both 3α-OH-DHP and 3α-THDOC are present in brain, as are their biosynthetic enzymes, 5α-steroid reductase and 3α-hydroxysteroid oxidoreductase (*18*). Moreover, 3α-OH-DHP is secreted by the adrenal, testis, and ovary, and both the blood and tissue concentrations of this steroid fluctuate during the estrous cycle in rats (*19*). Because 3α-THDOC is a major metabolite of deoxycorticosterone in man, and its secretion increases after infusion of ACTH (*20*), this steroid may participate in the CNS response to stress. Thus, 3α-OH-DHP and 3α-THDOC may function as endogenous barbiturate-like modulators of GABA-mediated synaptic inhibition in various physiological states.

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25. Crude synaptosomes (P_2 fraction) were prepared from the whole cerebral cortex of adult male Sprague-Dawley rats (150 to 200 g) (*21*). Whole forebrain was dissected on ice (0° to 4°C) and homogenized with a teflon-glass homogenizer in 15 volumes (weight to volume) 0.32*M* sucrose (0° to 4°C) containing 5 m*M* tris-HCl (*p*H 7.4). The homogenate was centrifuged at 1000*g* for 20 minutes and the resulting supernatant was centrifuged at 19,000*g* for 15 minutes to yield the crude synaptosomal pellet. The synaptosomal fraction was resuspended in 20 volumes of oxygenated Krebs-phosphate buffer containing 10 m*M* glucose (*p*H 7.4).
26. The apparent K_d values were 47.7 ± 2.3 (SEM) n*M*, 58.0 ± 2.6 n*M*, and 66.7 ± 3.5 n*M* (*) for control, 3α-THDOC (300 n*M*), and 3α-OH-DHP (300 n*M*), respectively. B_{max} values were 1156 ± 22, 997 ± 27 (*) and 836 ± 26 (*) fmol per milligram of protein for control, 3α-THDOC (300 n*M*), and 3α-OH-DHP (300 n*M*), respectively. The asterisk indicates that the value is statistically different from control, $P < 0.05$, Dunnett's test.
27. The apparent K_d values were 25.2 ± 1.6 (SEM) n*M*, 16.6 ± 0.7 n*M* (*), and 15.3 ± 0.5 n*M* (*) for control, 3α-THDOC, and 3α-OH-DHP, respectively. The asterisk indicates that the values are significantly different from control, $P < 0.05$, Dunnett's test. B_{max} values (approximately 3.85 pmol per milligram of protein) were apparently unchanged by steroids.
28. Synaptoneurosomes were prepared from cerebral cortex of adult male Sprague-Dawley rats (150 to 200 g) by homogenization by hand (in a glass-glass homogenizer) (*14*). The homogenate was filtered through three layers of nylon mesh and subsequently through a 10-μm Millipore filter. The filtrate was centrifuged at 1000*g* for 15 minutes, the supernatant was discarded, and the pellet was resuspended in buffer (containing 20 m*M* Hepes-tris, 118 m*M* NaCl, 4.7 m*M* KCl, 1.18 m*M* $MgSO_4$, and 2.5 m*M* $CaCl_2$, *p*H 7.4) at a concentration of 20 mg of protein per milliliter (*14*).
29. In this experiment 3α-OH-DHP significantly stimulated $^{36}Cl^-$ uptake at concentrations from 300 n*M* to 3 μ*M*. In some experiments a significant stimulation of $^{36}Cl^-$ uptake was observed at 3α-OH-DHP concentrations as low as 100 n*M*. Similar results were observed with 3α-THDOC.
30. Recordings in (A_1) and (A_2) were made at 22°C from rat hippocampal neurons grown in dissociated culture for 2 to 4 weeks (*22*). The whole-cell patch recording technique (*23*) was used, with patch pipettes of resistance 3 to 5 megohm filled with 145 m*M* potassium gluconate, 0.1 m*M* $CaCl_2$, 1.1 m*M* EGTA, 2 m*M* $MgCl_2$, and 5 m*M* Hepes-NaOH, *p*H 7.2. The osmolarity was adjusted by addition of sucrose to 315 mosmol. The extracellular solution contained 145 m*M* NaCl, 2 m*M* $CaCl_2$, 2 m*M* $MgCl_2$, 5 m*M* Hepes-NaOH, *p*H 7.4. Pipette current and voltage were recorded with a Ag-AgCl wire and List EPC-7 amplifier and displayed on a chart recorder. Action potentials in (A_2) are truncated due to the slow frequency response of the pen recorder.
31. Recordings in (B_1) and (B_2) were made as for (A_1) and (A_2) except that the extracellular solution contained 1 μ*M* tetrodotoxin, and the intracellular solution contained 145 m*M* CsCl in place of 145 m*M* potassium gluconate. This solution was used for voltage-clamp studies for two reasons. (i) Intracellular Cl^- loading results in an equilibrium potential for Cl^- ions of 0 mV; thus, at a holding potential of −60 mV, there is a 60-mV driving force for GABA-evoked Cl^- efflux. (ii) Intracellular Cs^+ blocks K^+ conductances in neurons (*24*). Both these factors contribute to a greatly improved signal-to-noise ratio for the GABA-activated Cl^- conductance. Data in (B_2) were sampled and digitized at 500 Hz with a PDP-11/23 computer, and stored for subsequent retrieval and display.

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