ETAZOLATE (SQ20009): ELECTROPHYSIOLOGY AND EFFECTS ON [³H]FLUNITRAZEPAM BINDING IN CULTURED CORTICAL NEURONS¹

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Abstract

The actions of etazolate (SQ20009) on cultured cortical neurons have been examined in electrophysiological experiments and in receptor binding studies. Etazolate (0.3 to 100 μ M) prolongs the duration of spontaneously occurring IPSPs. Higher concentrations of etazolate produce an increase in membrane chloride conductance, an effect which is picrotoxinin and bicuculline sensitive. Etazolate potentiates the response to exogenously applied GABA and acts synergistically with diazepam to enhance GABA-mediated conductance. Etazolate does not increase glycine-mediated conductance changes. Etazolate increases [³H]flunitrazepam binding and stimulates the GABA enhancement of [³H]flunitrazepam binding. In addition, GABA stimulates the etazolate enhancement of [³H]flunitrazepam binding. The etazolate-mediated increases in binding are Cl⁻ dependent and picrotoxinin and bicuculline sensitive. The dose response relationships for etazolate-mediated effects are similar in physiological experiments and in binding studies.

These data suggest that etazolate interacts with the postsynaptic GABA receptor complex at a site distinct from either the GABA recognition site or the benzodiazepine binding site to enhance GABA-mediated inhibition and to increase benzodiazepine binding.

Etazolate (SQ20009) is a compound that has been shown to have anxiolytic-like properties in rats (Beer et al., 1972; Horovitz et al., 1972) and to interact with the GABA receptor complex. In receptor binding studies utilizing membrane preparations of mammalian CNS, low concentrations of etazolate enhance benzodiazepine binding (Supavilai and Karobath, 1979; Williams and Risley, 1979; Leeb-Lundberg et al., 1981), stimulate Na⁺-independent GABA binding (Placheta and Karobath, 1980), and increase the GABA enhancement of benzodiazepine binding (Supavilai and Karobath, 1980; Leeb-Lundberg et al., 1981). The etazolate enhancement of benzodiazepine binding is Cl⁻ dependent (Supavilai and Karobath, 1979, 1981) and bicuculline (Williams and Risley, 1979; Leeb-Lundberg et al., 1981; Supavilai and Karobath, 1981) and picrotoxinin sensitive (Leeb-Lundberg et al., 1981; Supavilai and Karobath, 1979, 1981). It has been suggested that the etazolate enhancement of

benzodiazepine binding is mediated through the component of the GABA receptor complex identified as the picrotoxinin/barbiturate binding site (Leeb-Lundberg et al., 1981; Olsen, 1981; Ticku and Davis, 1982).

In order to understand the physiological actions of etazolate more completely, we have investigated its effects on the electrophysiological characteristics of neurons in primary cultures of rat cerebral cortex and its ability to modify GABA-mediated inhibition in these cells. In addition, we have evaluated the effects of etazolate on benzodiazepine binding and on the GABA stimulation of benzodiazepine binding in the same culture system. The use of cortical cultures has allowed us to study the actions of this drug on a living preparation by both physiological and biochemical methods. The dose response relationships and pharmacological specificities identified from the results of the physiological and biochemical studies are similar. These data indicate that etazolate increases GABA-mediated inhibition by prolonging the duration of IPSPs and suggest that etazolate interacts with a component of the GABA receptor complex which is distinct from either the GABA recognition site or the benzodiazepine binding site.

Materials and Methods

Tissue culture. Primary cultures of rat cerebral cortex were produced according to previously reported methods

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(Dichter, 1978; Snodgrass et al., 1980; White et al., 1981). Cerebral cortices were removed from fetal rats (15-day gestation) and were dissociated by mincing, trypsinization, and trituration. The dissociated cortical cells were plated onto either collagen-polylysine-coated coverslips maintained in 35-mm plastic dishes (for electrophysiology, 3.5×10^5 cells/dish) or into trays of similarly coated 16-mm wells (for biochemistry, 1×10^5 cells/well). The culture medium was Eagle's Minimal Essential Medium supplemented with 5% rat serum, 100 mg% glucose, penicillin (20 units/ml), and streptomycin (20 μ g/ml). The cells were kept in a 5% CO₂ incubator at 37°C, and the medium was changed three times per week. At confluence of non-neuronal cells, further mitosis was inhibited by the addition of 10^{-7} M cytosine arabinoside for 24 hr. Electrophysiological and biochemical assays were performed on cells after 3 to 7 weeks in culture.

Electrophysiology. Electrophysiological methods have been reported previously (Dichter, 1978, 1980). Coverslips containing cortical neurons were placed into 35-mm dishes containing a physiological salt solution (151 mM Na⁺, 3 mM K⁺, 154 mM Cl⁻, 1.8 mM Ca²⁺, 1 mM Mg²⁺, buffered with 2 mM phosphate to pH 7.2 to 7.3) and were maintained at 35° to 37°C. Individual neurons were visualized with an inverted phase microscope (Zeiss) at $\times 200$ magnification and were impaled with 3.5 M KAc-filled glass microelectrodes (35 to 85 megohms). The compounds under study were applied by pressure ejection from microelectrodes with tips broken to a diameter of approximately 10 μ m (Choi, 1978). In general, three drug-filled minipipettes were positioned near the impaled cell. Cell responses were analyzed from strip chart recordings and from photographed records of oscilloscope traces. Membrane input resistance was calculated by applying Ohm's law to the voltage changes which resulted from the injection of 50-msec hyperpolarizing constant current pulses (0.2 to 1.0 nA). Cells accepted for data analysis had resting membrane potentials of -40 mV or greater, initial input resistances of 30 to 100 megohms, and action potentials of 50 mV or greater.

Receptor binding studies. The methods employed for the binding studies were similar to those described previously (White et al., 1981). Cultures were washed three times with 1 ml of HEPES-buffered physiological salt solution (White et al., 1981) at room temperature, ~23°C, and were incubated for 15 min in 1 nM [³H]flunitrazepam and any other drug under study. Nonspecific binding was determined in parallel incubations containing 1 μ M clonazepam, a ligand which blocks binding to neuronal benzodiazepine receptors, and accounted for about 25% of the total binding. The incubation was terminated by two rapid (less than 8 sec total) washes in HEPES buffer. In studies employing Cl⁻-free media, all of the chloride was replaced by acetate. At the conclusion of the incubation and washes, the cultures were solubilized in 0.1 N NaOH. The amount of radioactivity in each sample was determined by liquid scintillation spectrometry using a Triton/toluene/Omnifluor liquid scintillation cocktail. All studies were performed in at least triplicate, and each study was replicated at least four times with cultures prepared from different dissections.

Materials. Etazolate was a gift from Squibb and Sons, Inc. (Princeton, NJ), and clonazepam was a gift from

Hoffman-La Roche, Inc. (Nutley, NJ). [³H]flunitrazepam was purchased from New England Nuclear. Bicuculline methiodide was purchased from Pierce Chemical Co. All other chemicals were of reagent grade and were purchased from Sigma.

Results

Electrophysiology

Etazolate effects on IPSP duration. Etazolate, at all concentrations tested (0.3 to 100 μ M), produced a large increase in the duration, but not the amplitude, of IPSPs (Fig. 1, A and B). The duration of IPSPs was measured as the time from onset to half-recovery (IPSP $t_{1/2}$). The IPSP $t_{1/2}$ values for control IPSPs varied from 10 to 25 msec and showed no consistent relationship to IPSP amplitudes (Fig. 1C). When etazolate was applied, IPSP $t_{1/2}$ values increased greatly and ranged from 30 to >100 msec. The increase in IPSP $t_{1/2}$ seemed to result from a prolongation in the decay of the IPSP. The rise time of the IPSP was rapid and did not seem to change with etazolate application. The change in IPSP $t_{1/2}$ occurred during etazolate application and persisted after drug perfusion was terminated (Fig. 1D). There was no consistent change in either EPSP duration or amplitude as a result of etazolate application.

Effects of etazolate on responses to exogenously applied GABA. It has been shown previously that the IPSPs in these cultures are GABA mediated and that virtually all the neurons are GABA sensitive (Dichter, 1980), findings which are in agreement with reports of *in situ* cortical sensitivity to GABA (for reviews see Krnjevic, 1974, and Curtis and Johnston, 1974). We tested the hypothesis that the effects of etazolate on IPSP duration were due to an interaction with the GABA receptor complex by examining the effects of etazolate on the response to exogenously applied GABA. The responses of a typical cell to the application of GABA alone, etazolate alone, and GABA + etazolate are shown in Figure 2 (see *insert*). The application of GABA (5 μ M) produced a moderate increase in membrane conductance, whereas etazolate (3 μ M) applied alone produced no conductance increase. When 3 μ M etazolate + 5 μ M GABA were applied, the increase in membrane conductance was ~3.5 times greater than that mediated by GABA alone. The etazolate-induced potentiation of the response to exogenously applied GABA suggests that this action of etazolate is mediated postsynaptically.

The dose response relationship for the etazolate potentiation of GABA-mediated conductance increases is shown in Figure 2. Low concentrations of etazolate (<1 μ M) produced no change in membrane conductance when applied alone and did not affect the GABA-mediated increase in membrane conductance. High concentrations of etazolate (10 to 100 μ M) caused membrane hyperpolarization and increased conductance. Etazolate (3 to 100 μ M) + GABA produced an increase in membrane conductance which was greater than the sum of the conductance changes due to etazolate and GABA alone. Thus, etazolate potentiated the response to exogenously applied GABA over a wide concentration range. The etazolate-mediated changes in membrane conductance for these studies were measured at the time point during the first 10 sec of drug perfusion which corresponded to

Figure 1. Effects of etazolate on IPSP duration in cultured cortical neurons. In this and subsequent electrophysiology figures, the illustrations are penwriter records of intracellular recordings. **A** and **B** show spontaneous postsynaptic potentials under control and drug-treated conditions. **A**, IPSPs in the same cell under control conditions and during the application of 1 μ M etazolate. Voltage responses are to 50-msec hyperpolarizing pulses of current. Membrane potential (-69 mV) and input resistance (58 megohms) are unchanged. **B**, Typical response of a neuron with spontaneous IPSPs to the first few seconds of perfusion with 30 μ M etazolate. The start of perfusion is marked by an arrow. Membrane potential was -49 mV throughout the record shown. This record is from a different cell than in **A** and is shown at a different chart speed than **A**. **C**, IPSP amplitude versus duration in four different cells to which 3 μ M etazolate was applied. Each point represents the amplitude (mV) and the $t_{1/2}$ (msec) of one IPSP either before ($\circ$) or during ($\bullet$) the application of etazolate. Twenty-nine IPSPs were evaluated prior to etazolate application, with a mean ($\pm$ SEM) $t_{1/2}$ of 12.9 ± 0.6 msec. Twenty-four IPSPs were evaluated during etazolate application with a mean $t_{1/2}$ of 28.1 ± 1.8 msec. **D**, Time course of the effects of 3 μ M etazolate on IPSP duration for the same cells as in **C**.

Figure 2. Summary of the effects of etazolate (ETAZ) on GABA-mediated changes in membrane conductance in synaptically active neurons. Solutions containing either 5 μ M GABA, 0.3 to 100 μ M etazolate, or GABA + etazolate were applied to cortical neurons as described under "Materials and Methods." Changes in membrane conductance for each cell are expressed in nanosiemens (nS) and are calculated from membrane input resistance at the time point which corresponds to the time of the maximal response to GABA + etazolate (see arrows over insert). Values represented in the histogram reflect individual neuronal responses to the first application of drug and are expressed as the mean conductance change in nS $\pm$ SEM. For each concentration of etazolate tested, the first bar represents the averaged change in conductance with GABA alone, the second bar shows the averaged response to etazolate alone, and the third bar indicates the averaged response when GABA + etazolate were applied. The number of cells tested at each concentration is given in parentheses. The responses of these neurons to the application of etazolate alone are included in the data shown in Figure 3. *Insert*, Response of a typical cell to 5 μ M GABA, 3 μ M etazolate, and 5 μ M GABA + 3 μ M etazolate. Bars over the traces represent the times during which drugs were applied. The following changes in membrane potential (mV) and in membrane conductance (nS) were observed: control = -44 mV, 22 nS; +GABA = -56 mV, 40 nS; +ETAZ = -47 mV, 20 nS; +GABA + ETAZ = -60 mV, 62 nS. (The membrane hyperpolarization occurring with etazolate application is not associated with a conductance increase and is probably an artifact of microperfusion.)

the maximal response to GABA + etazolate (see arrows in Fig. 2 insert).

Direct membrane effects of etazolate. The direct membrane effects of etazolate (3 to 100 μ M) in neurons with incoming synaptic activity are summarized in Figure 3. Concentrations of etazolate greater than 3 μ M produced a hyperpolarization which was maximal during the first 10 sec of drug perfusion (see insert, Fig. 3, point A) and a conductance change which continued for many seconds and was often maximal after etazolate perfusion had been terminated (see insert, Fig. 3, point B). The increases in membrane conductance were measured at the points of maximum drug effect and are summarized in Figure 3. Thirty micromolar etazolate produced maximal conductance increases, and the response diminished with 100 μ M etazolate.

Etazolate produced direct membrane effects (hyperpolarization and conductance increase) which were qualitatively similar to those induced by GABA. It has been shown previously that GABA increases Cl^- permeability in cultured cortical neurons (Dichter, 1980). We explored the possibility that etazolate also activated Cl^- channels in a simple experiment utilizing KAc and KCl electrodes and concentrations of etazolate (30 μ M) and GABA (60 μ M) which caused maximal effects. KCl electrodes allowed chloride ions to leak into the impaled cell and dramatically altered the Cl^- equilibrium potential. When

etazolate and GABA were applied alternately to a small group of neurons in which the internal chloride ion concentration had been changed, the membrane potentials produced by each compound showed a close correlation (Fig. 4). Therefore, it seemed likely that etazolate, like GABA, produced an increase in membrane permeability to Cl^- .

Effects of etazolate under conditions of reduced synaptic activity. As indicated above, etazolate potentiated the responses to exogenously applied GABA, and GABA and etazolate produced similar membrane effects. These data suggest that the direct effects of etazolate might result from an enhancement of the actions of endogenous GABA released into the culture medium. We investigated this possibility by blocking neuronal activity with tetrodotoxin (TTX) to decrease the spontaneous release of GABA. Etazolate (3 to 100 μ M) applied alone to TTX-treated cells produced smaller conductance increases than those observed under control conditions (Fig. 3). In addition, in TTX-treated cells, there was a trend toward a continued increase in membrane conductance at 100 μ M etazolate. These data suggest that reducing the amount of "endogenous" GABA decreased the etazolate response, an indication that at least some of the direct membrane effects of etazolate might be due to the potentiation of endogenous GABA.

It also seemed likely that endogenous GABA affected

Figure 3. Summary of the direct effects of etazolate on membrane conductance in synaptically active cells and in TTX-treated cells. Etazolate (3 to 100 μM) was applied to cortical neurons in solutions containing either physiological saline (PS) (■—■) or saline to which TTX had been added (□—□), as described under "Materials and Methods." Changes in membrane conductance (nS) were calculated from membrane input resistance values measured at the point of maximum conductance increase. Each point represents the mean nS $\pm$ SEM of individual neuronal responses to the first application of etazolate. The number of cells evaluated at each concentration of etazolate is given in parentheses. *Insert.* Typical response of a TTX-treated cell to 30 μM etazolate. Arrow at A indicates the point of maximal hyperpolarization; arrow at B indicates the point of the maximum conductance increase. Membrane potential (mV) and membrane conductance (nS) were as follows: control = -48 mV, 44 nS; etazolate = -57 mV, 66 nS.

the degree to which etazolate potentiated the conductance increase mediated by exogenously applied GABA. In order to evaluate this possibility, we applied etazolate + GABA to TTX-treated neurons and to cells bathed in 0 mM Ca^{2+} , 10 mM Mg^{2+} media. Under these conditions of reduced synaptic activity, etazolate (3 to 100 μM) greatly potentiated GABA responses, and the maximum enhancement occurred with 30 μM etazolate (Fig. 5). The combined GABA + etazolate responses were much greater in both TTX-treated cells and in cells bathed in low Ca^{2+} , high Mg^{2+} than in cells assayed under normal recording conditions (Fig. 2). These data suggest that, in addition to its effects on the "direct" response of etazolate, endogenous GABA may reduce the etazolate potentiation of responses to exogenously applied GABA. However, the mechanism by which this occurs is not clear.

Physiological interactions of etazolate with diazepam. Diazepam has been shown previously to increase IPSP amplitude and to enhance GABA-mediated conductance but to have no effect on conductance when applied alone (White et al., 1981). In order to study the physiological interactions of diazepam and etazolate, we examined the effects of each drug and of both together on spontaneously occurring IPSPs and on the conductance increases mediated by exogenously applied GABA.

Figure 4. Correlation of etazolate and GABA effects on membrane potential. Etazolate (30 μM) and GABA (60 μM) were applied alternately to 10 different neurons (mean resting membrane potential = -57 ± 13 mV), and the membrane potential produced by each compound was noted. Cells were impaled with either KAc (■) or KCl (●) electrodes to change internal chloride ion concentrations. Each point represents the intersection of the membrane potential values produced by the first application of etazolate and GABA in each cell. Line was drawn using linear regression; $r = 0.98$.

We chose a concentration of etazolate (3 μM) which prolonged the duration of IPSPs, potentiated the GABA-mediated increase in membrane conductance (Fig. 2), and did not change Cl^- conductance when applied alone (Fig. 3). The effects of 3 μM etazolate and 100 nM diazepam on IPSPs are illustrated in Figure 6A. Diazepam increased the amplitude but not the duration of inhibitory potentials, and etazolate increased the duration but not the amplitude. When etazolate and diazepam were applied together, both the amplitude and the duration of IPSPs were enhanced. There was no change in membrane conductance when either drug was applied alone nor when the two compounds were applied together.

The effects of etazolate and diazepam on the response to exogenously applied GABA are shown in Figure 6B. When etazolate + diazepam + GABA were applied, the enhancement of the GABA-mediated increase in Cl^- conductance was greater (average of 50 to 60% greater in seven cells) than the sum of the individual drug potentiations. Etazolate and diazepam seemed to interact with the GABA receptor complex in different ways, because the two drugs had different effects on IPSPs and were synergistic in their enhancement of GABA-mediated increases in Cl^- conductance.

Pharmacological specificities of etazolate effects. The pharmacological specificities and sensitivities of the physiological actions of etazolate were investigated using

Figure 5. Summary of the effects of etazolate on GABA-mediated changes in membrane conductance in TTX-treated cells. Physiological saline + TTX and either 5 μ M GABA, 3 to 100 μ M etazolate, or GABA + etazolate were applied to cortical neurons as described under "Materials and Methods." Changes in membrane conductance (nS) were measured as in Figure 2, at the time point which corresponded to the time of the maximum response to GABA + etazolate. Values reflect individual neuronal responses to the first application of drug and are expressed as the mean conductance change in nS $\pm$ SEM. For each concentration of etazolate tested, the *first bar* represents the averaged conductance changes with GABA, the *second bar* shows the averaged responses to etazolate, and the *third bar* shows the averaged responses to GABA + etazolate. The number of cells tested at each concentration is given in parentheses. The responses of these neurons to the application of etazolate alone are included in the data shown in Figure 3 and are not significantly different from the data given in that figure.

agents which interact with the GABA receptor complex. Picrotoxinin is a noncompetitive antagonist which blocks the GABA-mediated increase in Cl^- conductance (Takeuchi and Takeuchi, 1969; for review see Obata, 1977) but acts at a site distinct from the GABA recognition site (Olsen et al., 1978); bicuculline is a competitive antagonist of the GABA recognition site (Johnston et al., 1972; Simmonds, 1980; for review see Andrews and Johnston, 1979). In our system, both 50 μ M picrotoxinin (Fig. 7A) and 50 μ M bicuculline (Fig. 7B) blocked the etazolate-mediated membrane hyperpolarization and conductance

increase which occurred during drug application. Interestingly, the maximum conductance increase produced by etazolate which typically occurred after the termination of drug perfusion was antagonized more by picrotoxinin than by bicuculline (indicated by arrows in Fig. 7). These data demonstrate that the actions of etazolate are sensitive to both picrotoxinin and bicuculline and suggest that there may be some differences in the interaction of these two GABA antagonists with etazolate.

Both GABA and glycine (Barker and Ransom, 1978; Barker and McBurney, 1979a; Dichter, 1980) have been shown to increase Cl^- conductance in cultured neurons. In order to test whether etazolate was specific for GABA receptor-associated Cl^- channels, we applied etazolate together with glycine in the same experimental paradigm used for GABA. No increase in the glycine response was observed ($n = 5$ cells), indicating that the etazolate enhancement of Cl^- conductance was due to a specific interaction with the GABA receptor complex.

Etazolate has been shown to be a phosphodiesterase (PDE) inhibitor in neuronal and non-neuronal systems (Beer et al., 1972; Mansour and Mansour, 1979), as is SQ20006 (Chasin et al., 1977), a structurally related compound which has no anti-anxiety activity. The effects of 10 μ M and 100 μ M SQ20006 on IPSPs and on GABA-mediated responses were examined ($n = 14$ cells). There was no increase in the duration of IPSPs when SQ20006 was applied alone, nor did this drug produce an increase in membrane conductance. The application of SQ20006 together with 5 μ M GABA did not result in any stimulation of GABA-mediated effects. These data suggest that the physiological effects of etazolate are probably not due to its activity as a PDE inhibitor.

Receptor binding studies

The binding of [^3H]flunitrazepam to the neuronal-type benzodiazepine binding site has been investigated extensively on living cultures of rat cerebral cortex (White et al., 1981). These studies demonstrated numerous interactions between the binding of [^3H]flunitrazepam and known components of the GABA receptor complex including the GABA recognition site, the picrotoxinin recognition site, and the Cl^- ionophore. It has also been shown that GABA increases [^3H]flunitrazepam binding with a dose response relationship which is similar to that observed for the physiological action of GABA (White et al., 1981). These results suggest that changes in benzodiazepine binding can be used to monitor interactions of a compound with the GABA receptor complex. In the present experiments we measured the binding of [^3H]flunitrazepam to living cultures to evaluate the effects of etazolate on the GABA receptor complex.

Effects of etazolate on [^3H]flunitrazepam binding to living cortical cultures. The dose response relationship for the effect of etazolate on [^3H]flunitrazepam binding is shown in Figure 8. Etazolate, at concentrations between 1 and 10 μ M, produced a dose-dependent increase in the binding of [^3H]flunitrazepam to $124 \pm 7\%$ of control. At higher concentrations this effect was reversed, and by 100 μ M there was a decrease in binding to $88 \pm 7\%$ of control. This inverted U-shaped dose response relationship was similar to that reported above for the physiological actions of this drug (Figs. 2, 3, and 5), although

Figure 6. Physiological interactions of etazolate (ETAZ), diazepam (DZP), and GABA. **A**, Effects of 100 nM DPZ and 100 nM DZP + 3 μM ETAZ on spontaneously occurring IPSPs in a single cell. The same chart speed is shown in all three traces. Control record shows 2.6 to 3.6 mV IPSPs. One hundred nanomolar DZP alone increases IPSP amplitude to 6.0 to 6.7 mV but does not affect duration. One hundred nanomolar DZP + 3 μM ETAZ increase both IPSP amplitude and duration. (See Figure 1, A and B for the effects of ETAZ alone on IPSPs.) Membrane potential (mV) and conductance (nS) are unchanged from control values: -60 mV, 9 nS. **B**, ETAZ (3 μM) and DZP (100 nM) potentiation of the GABA (5 μM)-mediated conductance increase. Response of a different cell than in A shown at a slower chart speed. Drug applications are indicated by bars over the traces. The following increases in membrane potential (mV) and membrane conductance (nS) were noted: +GABA = -9 mV, 23 nS; +GABA + DZP = -11 mV, 32 nS; +GABA + ETAZ = -13 mV, 61 nS; +GABA + DZP + ETAZ = -14 mV, 132 nS.

the exact concentrations of etazolate which produced maximal effects differed in electrophysiological and in binding studies. The data shown in Figure 8 parallel very closely those reported by others for the action of etazolate on benzodiazepine binding in membrane preparations of CNS (Williams and Risley, 1979; Leeb-Lundberg et al., 1981).

Interactions of etazolate with GABA. Figure 9 illustrates the dose response relationship for the etazolate enhancement of [³H]flunitrazepam binding in the presence and absence of 10 μM GABA. In either case, low concentrations of etazolate (≤10 μM) enhanced [³H]flunitrazepam binding, and high concentrations (30 to 100 μM) decreased binding. In the presence of 10 μM GABA, the threshold for etazolate enhancement was decreased from 1.0 μM to less than 0.1 μM, and the magnitude of the enhancement was greater than that observed with etazolate alone. These results suggest that GABA and etazolate act synergistically to enhance the binding of [³H]flunitrazepam.

The dose response relationship for GABA-mediated stimulation of [³H]flunitrazepam binding in the presence and absence of 3 μM etazolate is presented in Figure 10. GABA stimulated [³H]flunitrazepam binding with a dose response relationship similar to that observed previously (White et al., 1981). Etazolate shifted the dose response curve for GABA to the left, indicating that it increased the efficacy with which GABA enhanced [³H]flunitrazepam binding. The presence of etazolate also caused a reversal in the enhancement of binding with higher concentrations of GABA (>10 μM). This reversal in the action of GABA was not seen in the absence of etazolate even at very high GABA concentrations (to 5 mM) tested previously (White et al., 1981). These results suggest an interesting interaction between GABA and etazolate. Either GABA (>10 μM) shifted the etazolate-induced decrease in benzodiazepine binding to lower concentrations of etazolate, or etazolate modified the action of high concentrations of GABA to produce a decrease in binding. The former explanation seems to be more likely, but

Figure 7. Pharmacological sensitivities of the etazolate response. Both *A* and *B* are shown at the same chart speed. Bars over the traces indicate drug applications. Arrows indicate points of maximum conductance increase. *A*, Response of a typical neuron to the application of 50 μ M picrotoxinin (PTX), 30 μ M etazolate (ETAZ), and PTX + ETAZ. Control membrane potential varied from -47 to -49 mV. *B*, Response of a different cell to the application of 50 μ M bicuculline (BIC), 30 μ M ETAZ and BIC + ETAZ. Membrane potential prior to the application of BIC and ETAZ was -71 mV and was -64 mV before the application of BIC + ETAZ. Current pulses were 0.37 nA in the BIC and ETAZ records and were 0.47 nA during the BIC + ETAZ record.

Figure 8. Dose response relationship for etazolate effects on $[^3\text{H}]$ flunitrazepam binding to cultures of rat cerebral cortex. Cultures were incubated with etazolate (10 nM to 100 μ M) and 1 nM $[^3\text{H}]$ flunitrazepam as described under "Materials and Methods." Each point represents the mean percentage ($\pm$ SEM) of specific binding over control values for quadruplicate determinations in four different experiments.

Figure 9. Effects of 10 μ M GABA on the etazolate potentiation of $[^3\text{H}]$ flunitrazepam binding to cortical cultures. Etazolate alone increased the binding of $[^3\text{H}]$ flunitrazepam over a concentration range of 1 to 10 μ M. The etazolate-mediated increase in binding was potentiated in the presence of 10 μ M GABA, and the threshold for the etazolate effect was decreased. The dotted line represents specific binding in the presence of 10 μ M GABA with no added etazolate. The results are expressed as a percentage of control specific binding, as described in Figure 8.

Figure 10. Effects of etazolate on the GABA potentiation of [^3H]flunitrazepam binding to cortical cultures. GABA (100 nM to 1 mM) potentiates specific binding in the absence of etazolate. In the presence of 3 μM etazolate, the dose response curve is shifted to the left, and with high concentrations of GABA there is decreased binding. The dotted line shows [^3H]flunitrazepam binding in the presence of 3 μM etazolate with no added GABA. The results are expressed as a percentage of control specific binding, as described in Figure 8.

it is difficult to distinguish between these possibilities given our current understanding of the GABA receptor complex. In either case, the data indicate that, at low concentrations, GABA and etazolate affect different but interacting sites to increase the binding of [^3H]flunitrazepam to cortical neurons.

Pharmacological sensitivities of etazolate interactions with the GABA receptor complex. Ligands for the other components of the GABA receptor complex modify the binding of [^3H]flunitrazepam to cultured neurons (White et al., 1981). In the absence of exogenous GABA, bicuculline slightly depresses the binding of [^3H]flunitrazepam and picrotoxinin has no effect; both antagonists block the GABA-mediated enhancement of [^3H]flunitrazepam binding. The replacement of Cl^- in the incubation media with acetate enhances [^3H]flunitrazepam binding and blocks the ability of GABA to enhance [^3H]flunitrazepam binding. In the studies reported here, we investigated the ability of etazolate to modify these interactions among the different components of the postsynaptic GABA receptor complex.

In the present studies, both bicuculline methiodide (100 μM) and picrotoxinin (100 μM) blocked the etazolate (10 μM) enhancement of [^3H]flunitrazepam binding (Table I). The replacement of chloride in the media by the impermeable anion acetate also blocked the etazolate enhancement of benzodiazepine binding (Table I). These results demonstrate that many of the ligands known to interact with the GABA receptor complex modified the actions of etazolate on benzodiazepine binding; GABA increased the enhancement, and the enhancement was blocked by bicuculline, picrotoxinin, and acetate.

TABLE I

Pharmacological specificities of etazolate potentiation of [^3H]flunitrazepam binding to cortical cultures

Data are expressed as a percentage of control specific binding (mean $\pm$ SEM).

	Chloride	Acetate
Etazolate, 10 μM ($n = 9$)	115 $\pm$ 5	87 $\pm$ 4
GABA, 10 μM ($n = 5$)	133 $\pm$ 8	111 $\pm$ 7
Etazolate, 10 μM + GABA, 10 μM ($n = 5$)	156 $\pm$ 10	117 $\pm$ 7
Bicuculline, 100 μM	82 $\pm$ 5	
Etazolate, 10 μM + bicuculline, 100 μM ($n = 3$)	83 $\pm$ 6	
Picrotoxinin, 100 μM ($n = 3$)	103 $\pm$ 3	
Etazolate, 10 μM + picrotoxinin, 100 μM ($n = 3$)	87 $\pm$ 7	

Discussion

The effects of etazolate (SQ20009) have been evaluated on cultured cortical neurons in electrophysiological experiments and in ligand binding studies. The following data indicate that the actions of etazolate are mediated through the postsynaptic GABA receptor complex: (1) etazolate prolongs the duration of IPSPs known to be GABAergic; (2) etazolate enhances GABA-mediated increases in membrane conductance; (3) etazolate stimulates the GABA enhancement of [^3H]flunitrazepam binding; (4) etazolate increases membrane conductance by increasing membrane permeability to chloride ions, an effect which is bicuculline and picrotoxinin sensitive; (5) etazolate potentiates benzodiazepine binding in a Cl^- -dependent manner which is also bicuculline and picrotoxinin sensitive; (6) etazolate and diazepam act synergistically to stimulate GABA-mediated increases in membrane conductance; (7) the etazolate stimulation of [^3H]flunitrazepam binding is potentiated by GABA; (8) etazolate does not enhance glycine-mediated conductance increases. Our data are completely consistent with the concept that the actions of etazolate are mediated through a postsynaptic locus. We did not see any alteration in action potential characteristics (size, duration, threshold, spontaneous frequency) which could have resulted in prolonged IPSP durations by a presynaptic mechanism.

Our data also suggest that etazolate interacts with the GABA receptor complex at a site which is distinct from either the GABA recognition site or the benzodiazepine binding site. The effects of low concentrations of etazolate and GABA are synergistic in both physiological experiments and in binding studies, implying different sites of action. The effects of etazolate and diazepam on spontaneously occurring IPSPs differ; etazolate prolongs duration, and diazepam increases amplitude. Etazolate and diazepam act in a greater than additive manner to enhance GABA-mediated conductance increases. The synergistic nature of etazolate-GABA-benzodiazepine interactions and the dissimilar effects produced by etazolate and diazepam on IPSPs are indications that these compounds act at functionally related but distinct sites.

The effects of etazolate in both physiological experiments and in ligand binding studies are similar to the effects of certain barbiturates. Etazolate, like phenobar-

bital (Barker and McBurney, 1979b) and pentobarbital (Nicoll et al., 1975), prolongs IPSP duration. Noise analyses of single channel kinetics indicate that both phenobarbital (Barker and McBurney, 1979b) and (-)pentobarbital increase the average open time of GABA-activated channels and that (-)pentobarbital decreases the frequency of their opening (Study and Barker, 1981). Thus, etazolate might also work by increasing the mean open time of Cl^- channels associated with the GABA receptor complex. Etazolate, like both phenobarbital and pentobarbital (MacDonald and Barker, 1979), potentiates GABA-mediated increases in membrane conductance. The inverted-U dose response curves presented here for the physiological and biochemical effects of etazolate are similar to those reported by others for the effects of etazolate on benzodiazepine binding (Williams and Risley, 1979; Supavilai and Karobath, 1981) and on GABA binding (Placheta and Karobath, 1980) and are also comparable to the dose response relationship reported for barbiturate enhancement of GABA binding (Willow and Johnston, 1981). In addition, the pharmacological sensitivities noted here for etazolate are similar to those reported for the physiological (MacDonald and Barker, 1979) and biochemical (Leeb-Lundberg et al., 1980, 1981) actions of the barbiturates. It seems likely that etazolate acts at the picrotoxinin/barbiturate binding site of the GABA receptor complex (Leeb-Lundberg et al., 1981; Olsen, 1981; Ticku and Davis, 1982) and that its mechanism of action resembles that of certain barbiturates.

In addition to etazolate and the barbiturates, the benzodiazepines also enhance GABA-mediated inhibition (Choi et al., 1977; MacDonald and Barker, 1978; White et al., 1981) and affect chloride channel activity (Study and Barker, 1981). The clinical effects of the barbiturates and the benzodiazepines are well documented, and include anticonvulsant, anesthetic, anxiolytic, and hypnotic actions (Gilman et al., 1980). Because etazolate shares many of their actions at the cellular level, it is reasonable to speculate that the behavioral effects of etazolate would also be similar. Etazolate has been shown to have anxiolytic-like activity in rats and, in the same studies, to increase the anti-anxiety effects of chlordiazepoxide (Beer et al., 1972; Horovitz et al., 1972). It is likely that etazolate also has other behavioral effects which are dependent upon its interactions with the GABA receptor complex, and these studies should be encouraged.

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