Diffential Regulation of \( \gamma \)-Aminobutyric Acid Receptor Channels by Diazepam and Phenobarbital

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The anticonvulsant activity of diazepam and phenobarbital may be mediated in part by enhancement of inhibition involving \( \gamma \)-aminobutyric acid (GABA). While both diazepam and phenobarbital increase GABA receptor chloride current, they may have different mechanisms of action, since they bind to different sites on the GABA receptor–chloride channel complex. We used the patch clamp technique to compare the effects of diazepam and phenobarbital on single GABA receptor currents. Outside-out patches were obtained from mouse spinal cord neurons grown in cell culture for 2 to 4 weeks. GABA (2 \( \mu \)M) evoked single channel currents that occurred as single brief openings or in bursts of multiple openings. Diazepam (20 \( \text{nM} \)) and phenobarbital (500 \( \text{pM} \)) both increased the GABA receptor current by increasing mean open time without altering channel opening frequency. However, the temporal grouping of openings into bursts suggested that the enhancement occurred via different mechanisms. Diazepam increased the frequency of bursting GABA receptor currents with minimal effect on the duration of bursts. Phenobarbital increased the duration of bursting GABA receptor currents without altering the frequency of bursts. These results suggest that diazepam binds to a site that may enhance single channel burst frequency by increasing the affinity of GABA binding, while phenobarbital may stabilize the bursting open state of the channel by binding to a different modulatory site at or near the chloride channel.


Benzodiazepine and barbiturate drugs are used clinically for their anticonvulsant, anesthetic, anxiolytic, and sedative-hypnotic effects [1]. The anticonvulsant activity of these structurally distinct classes of drugs may, in part, be mediated by potentiating \( \gamma \)-aminobutyric acid (GABA)-mediated inhibition of neuronal excitability [2–5]. The postsynaptic GABA\(_A\) receptor complex contains a chloride channel as well as binding sites for GABA. In addition, the receptor contains specific binding sites for benzodiazepines and barbiturates [4]. The GABA receptor is structurally composed of two polypeptide subunits, \( \alpha \) and \( \beta \) chains, with a subunit structure of \( \alpha_2\beta_2 \). The benzodiazepine binding site is located on the \( \alpha \) chain, while the GABA binding site is located on the \( \beta \) chain [6]. It is unclear where the barbiturate binding site is located. The benzodiazepine diazepam (DZ) binds at the benzodiazepine binding site and enhances binding of GABA. The barbiturate phenobarbital (PhB) binds at or near the chloride channel but does not significantly affect binding of GABA [5, 7]. The physiological consequence, however, of the different binding sites for these two anticonvulsant drugs is unclear.

GABA binds to its receptor and gates open the chloride channel, producing an inward chloride ion flux and membrane hyperpolarization. Benzodiazepines and barbiturates increase and/or prolong GABA receptor current, but the mechanisms of drug modulation of coupling between neurotransmitter binding and chloride channel gating are poorly understood. Characterization of drug effects on channel gating has not been possible with intracellular and noise analysis techniques. Recent development of the patch clamp technique, however, has enabled direct recording of neurotransmitter-gated single chloride channel currents [8]. Patch clamp studies of the nicotinic acetylcholine (ACh) receptor complex have revealed that binding of ACh increases the likelihood that the ACh receptor channel will open in bursts or groups of openings separated by relatively long closed periods [9]. Direct recording of channel openings and closings allows statistical inferences to be made about the properties of neurotrans-
mitter-gated channels and modification of that gating by drugs. Thus, it is now possible to study directly the coupling between neurotransmitter binding and channel gating and the actions of modulatory drugs on the channel gating process.

We have used the patch clamp technique to investigate the actions of DZ and PhB on single GABA receptor chloride currents from mouse spinal cord neurons grown in dissociated cell culture [10-12]. GABA responses of mouse spinal cord neurons have been shown to be enhanced by benzodiazepines and barbiturates and antagonized by the GABA antagonists picrotoxin and bicuculline [13]. In this study we have shown that DZ and PhB enhance GABA receptor single channel chloride currents by different mechanisms. These results provide evidence at the molecular level for different modes of action for the regulation of GABA receptor channels by benzodiazepines and barbiturates, which suggest that the modulatory sites for these drugs at the GABA receptor are different.

Methods
Cultures were prepared from spinal cords obtained from 12- to 14-day-old fetal mice as previously described [3, 10]. Briefly, a single-cell suspension obtained after mechanical dissociation and trypsinization was plated on collagen-coated dishes at a density of 10^6 cells/ml in a growth medium containing 80% minimal essential medium, 10% fetal calf serum, and 10% heat-inactivated horse serum. Following 3.5 days of incubation, uridine (15 pg/ml) and 5'-fluorodeoxyuridine (15 pg/ml) were added to suppress growth of rapidly growing nonneuronal cells and the medium was changed to 90% minimal essential medium and 10% heat-inactivated horse serum. Cultures were maintained for 2 to 4 weeks prior to use.

At the time of recording, culture medium was exchanged for a physiological salt solution (in mM: 142 NaCl, 8.09 KCl, 1 CaCl_2, 6 MgCl_2, 10 glucose, 10 Na-HEPES [pH 7.4]) containing strychnine (200 nM, Sigma Chemical Co, St Louis, MO), a specific glycine antagonist. Tetrodotoxin (1 μM, Sigma Chemical Co) was added to the solutions in the whole cell recordings to suppress spontaneous activity. Recording patch clamp pipettes were filled with a salt solution (in mM: 153 KCl, 1 MgCl_2, 10 Na-HEPES, 5 ethylene bis(oxyethylenenitrilo)tetraacetic acid, 1 NaOH, 1 KOH [pH 7.38]) to provide a symmetrical chloride concentration (chloride equilibrium potential 0 mV).

A local pressure ejection technique was used to apply GABA (Sigma Chemical Co) and the drugs to the neurons or excised outside-out patches. GABA alone or with DZ or PhB was dissolved in bath solution at the desired concentration and used to fill a blunt-tipped micropipette. To apply test solutions, a tight-fitting polyethylene tube was fitted over the open end of the pipette and connected to a regulated pressure source. GABA (2 μM) was applied by pressure ejection for 20 to 30 seconds. GABA (2 μM) and DZ (20 nM, Hoffmann-LaRoche, Nutley, NJ) or GABA and PhB (500 μM, Sigma Chemical Co) were applied by pressure ejection, and DZ (20 nM) and PhB (500 μM) were either pressure applied or added to the bath prior to exposure to GABA plus anticonvulsant. Pressure was applied to ejection pipettes for 5 to 10 seconds when the pipettes were distant from the cell or patch to ensure that no dilution of drug had occurred at the pipette tip. The tips of the pressure ejection pipettes were then brought to within 50 μm of the neuron or patch for application and were removed immediately from the bath solution after drug application.

Whole cell and patch clamp recording techniques [8] were similar to those of Hamill and colleagues [15]. Neurons or membrane patches were voltage clamped at the potassium equilibrium potential (-75 mV). Recordings were obtained using an I/M EPC-7 patch clamp amplifier (List Medical Instruments, Darmstadt, West Germany) and recorded on a video data recording system for later analysis. Single-channel data were digitized at 8 kHz with a 1-kHz eight-pole Bessel filter interposed. Channel openings and closings of the main conductance state (27-30 picosecond [pS]) were detected using a 50% threshold crossing criterion [8] and analyzed using programs written in this laboratory. Only patches with stable responses and infrequent multiple simultaneous channel openings were used for analysis. Those rare multiple openings detected by the program were classified as closures. Events less than twice the dead time of the system (dead time = 130 μsec) were rejected from analysis.

Since channels open and close randomly with a characteristic probability, several thousand events must be obtained to evaluate their probabilistic (nongaussian) distribution. However, to compare the effects of drugs on the GABA receptor channel openings, data were analyzed by calculating the mean open, closed, and burst durations (defined below) per GABA application and evaluating the results by a Student's t test for independent groups. Data were presented in the form of mean ± standard error unless otherwise specified. To display frequency distributions, open and closed durations were pooled from several recordings and collated into frequency histograms of variable binwidths.

Results
Whole-cell inward chloride currents exhibited typical GABA pharmacological characteristics and were blocked by picrotoxin and bicuculline (not illustrated). Whole-cell recordings were used to establish DZ and PhB concentrations that produced similar enhancement of GABA receptor currents. GABA evoked inward current with an associated increase in membrane conductance (Fig 1A1, B1). Both DZ and PhB produced about a twofold increase in the GABA receptor current (Fig 1A2, B2). The enhancement of GABA receptor current was reversible (not illustrated).

Single channel chloride currents exhibited typical GABA pharmacological characteristics and were blocked by picrotoxin and bicuculline (not illustrated). Excised outside-out patches that demonstrated GABA-sensitive channel openings typically had rare, brief spontaneous openings (Fig 2A). GABA (2 μM) evoked predominantly bursting currents (Fig 2B)
80% of patches. Openings with at least two different current amplitudes were recorded, representing two different conductance states (not illustrated). The larger conductance state (approximately 26–30 pS) occurred much more frequently and was used in these analyses. DZ (20 nM) and PhB (500 µM) both increased single channel activity evoked by GABA (Fig 2C,D). Channel conductance in the presence of DZ and PhB was unchanged. A drug may increase ion flux through a channel by independent or combined increases in channel conductance, open duration, or opening frequency. Since neither DZ nor PhB altered conductance, we determined the opening and closing temporal characteristics of the channel.

Multiple single-channel recordings of GABA receptor single channel currents were summed to produce an average current per recording. The time course of the summed single channel GABA receptor current (Fig 3A) was similar to the GABA-evoked whole cell current (see Fig 1). Both DZ and PhB enhanced the summed single channel GABA receptor currents (Fig 3B,C). For both anticonvulsants, the enhanced, summed currents had a time course similar to that for the whole cell currents (see Fig 1). In the early portions of the summed and whole cell currents, current increased. This was likely due to the lag time before the concentration of GABA from the ejection pipette became maximal and constant. In the presence of DZ and PhB, the current tended to decline over time, possibly because of desensitization. However, the currents were relatively stable after the initial rising period and prior to the late decline of current. For analysis of the temporal characteristics of the current, it was important that the current was stable or stationary. Therefore, for kinetic analysis of single channel gating characteristics, currents were analyzed from 2 to 17 seconds after the first channel opening.

Both DZ and PhB altered the mean open properties of GABA receptor single channel currents (Table). Mean open time per application of GABA was 5.0 msec (158 applications). DZ increased the mean open time to 7.3 msec ($p < 0.0005$, 22 applications). PhB also increased the mean open time to 5.7 msec ($p < 0.05$, 54 applications). Per application, GABA evoked a mean of 13.4 openings/sec. Neither DZ (12.9 openings/sec, not significant (NS)) nor PhB (15.5 openings/sec, NS) altered the number of GABA receptor openings per second. With GABA applied alone, the channel was open 6.2% of analysis time. Following the addition of DZ or PhB, the channel was open 8.9% ($p < 0.05$) and 8.4% ($p < 0.0025$) of analysis time, respectively. Thus, DZ increased the mean open duration for each channel opening but did not increase the frequency of opening. Although PhB was less effective in increasing mean open duration, the slight increase in GABA receptor channel opening frequency accounted for approximately the same total increase in ion flux.

To visualize how DZ or PhB increased the mean open duration, channel open or closed durations may be displayed in the form of histograms showing the relative frequency of occurrence of an opening or closing with a particular duration. In general, the longer an opening or closing, the less frequently it was observed. Frequency distribution histograms were obtained and normalized to display the effects of the drugs on the distributions. Visual inspection of normalized open duration frequency histograms showed that in the presence of DZ or PhB, mean channel open time was...
Fig 2. Single channel chloride current evoked by γ-aminobutyric acid (GABA) was enhanced by diazepam (DZ) and phenobarbital (PhB). (A) Prior to application of GABA, only rare brief spontaneous currents were present. (B) Following application of GABA, channel openings increased in frequency and occurred as individual openings or in groups of openings (bursts). (C) In the presence of DZ, GABA produced increased channel activity. (D) In the presence of PhB, GABA again increased GABA receptor channel activity. Membrane patches were voltage clamped at the potassium equilibrium potential (-75 mV). Downward deflections in all tracings represent channel openings and inward chloride currents.

increased (Fig 4). DZ shifted the distribution of openings to longer durations but appeared to have a greater effect on increasing the relative proportion of long openings. The effect of PhB was a shift of the distribution of all openings to longer durations. In particular, PhB had a greater effect than DZ had on the distribution of short openings. The effect of DZ on the relative proportion of long openings was also apparent in that the mean open time for DZ was longer than that for PhB. Although closed times varied over a wide range (from microseconds to seconds), the distributions of closed time less than 50 msec were altered little by DZ or PhB (Fig 5).

The general properties of mean open duration and opening frequency do not provide information about how openings may be clustered or grouped together. A burst can be defined as a group of openings separated by a relatively long closure [8]. As previously mentioned, both GABA and ACh evoke bursts of channel openings, suggesting that bursting activity may be a fundamental behavior of bound receptor-gated ion channels. The bulk of the ion flux through the channel occurs during a burst containing several openings, whereas isolated, brief channel openings pass relatively little current. To examine burst characteristics in this analysis, only those events longer than 10 msec were evaluated. Thus, a series of openings and closings

Fig 3. Diazepam (DZ) and phenobarbital (PhB) increased γ-aminobutyric acid (GABA) receptor current. (A) GABA was applied to 115 patches to evoke inward single channel currents. The currents from these patches were added and resulted in a summed inward current. This summed inward current had a time course similar to that of the inward currents produced by GABA using whole cell recording. (B) Single channel recordings from 22 patches in the presence of GABA and DZ were added to produce a summed single channel current. The current was normalized for the number of patches for comparison to A. (C) Multiple recordings of single channels from 54 patches in the presence of GABA and PhB were added to produce a summed current. The summed current was then normalized to the number of patches for an ensemble average current for comparison to A. In all cases, each application of GABA was added from the beginning of the first opening. Multiple simultaneous channel openings were not rejected so that total charge transfer per application could be added. These summed traces demonstrate that DZ and PhB enhanced the total amount of current passed per application of GABA.
GABA Receptor Channel Open Properties

<table>
<thead>
<tr>
<th></th>
<th>GABA</th>
<th>GABA + Diazepam</th>
<th>GABA + Phenobarbital</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean open time (msec)</td>
<td>5.0 ± 0.2</td>
<td>7.3 ± 0.6***</td>
<td>5.7 ± 0.4**</td>
</tr>
<tr>
<td>Openings per second</td>
<td>13.4 ± 1.0</td>
<td>12.9 ± 1.9</td>
<td>15.5 ± 2.1</td>
</tr>
<tr>
<td>Percent time channel open</td>
<td>6.2 ± 0.5</td>
<td>8.9 ± 1.5**</td>
<td>8.4 ± 1.1*</td>
</tr>
<tr>
<td>Total number of openings</td>
<td>28,872</td>
<td>4,051</td>
<td>10,400</td>
</tr>
<tr>
<td>Number of drug applications</td>
<td>158</td>
<td>22</td>
<td>54</td>
</tr>
</tbody>
</table>

*To maintain stationary channel kinetics, single channel records were analyzed from 2 to 17 seconds after GABA or GABA plus drug application. Data are presented in the form of mean ± standard error per application of drug. GABA, diazepam, and phenobarbital concentrations were 2 µM, 20 nM, and 500 µM, respectively.

bThe percentage of time in the recording from 2 to 17 seconds that the channel was in the open state.

*p < 0.05.

**p < 0.005.

***p < 0.0005.

GABA = γ-aminobutyric acid.

Fig 4. Normalized channel open time frequency histogram. Frequency histograms display the relative occurrence of an opening or closing of a particular duration. In general, a long duration event occurred less frequently than a short one. In the presence of diazepam (DZ) (B) and phenobarbital (PhB) (C), the distribution of channel open time was shifted to longer open times compared with γ-aminobutyric acid (GABA) alone (A). Frequency histogram range is 0.0 to 50.0 msec and the binwidth is 0.5 msec. The number of openings contained in each histogram were as follows: GABA = 42,136; GABA + DZ = 12,976; GABA + PhB = 21,158.

Fig 5. Normalized channel closed time frequency histogram. Closed times varied over a wide range but are displayed only from 0.5 to 50.0 msec with a 0.5 msec binwidth. The distributions were not overlapping for γ-aminobutyric acid alone and with diazepam and phenobarbital.

GABA evoked bursts with a mean duration per application of 65.8 ± 5.2 msec (158 applications). In the presence of DZ, mean GABA receptor burst duration was increased by 9.3% (71.9 ± 7.0 msec, NS, n = 22), but frequency of GABA-receptor bursts was increased by 102.5 ± 6.5% (p < 0.0001) (Fig 6). In contrast, in the presence of PhB mean GABA receptor burst duration was prolonged by 81.9% (119.7 ± 16.1 msec, p < 0.0001, n = 54), but GABA receptor burst frequency increased only by 6.3 ± 5.3% (NS) (see Fig 6). Thus, the mechanism of potentiation of GABA receptor current by DZ appeared to be mediated by increasing the likelihood or frequency of bursts evoked by GABA without altering the burst duration. PhB, on the other hand, potentiated GABA receptor current by prolonging bursts without altering frequency. These burst properties indicate fundamentally different mechanisms of the regulation of GABA receptor chloride channel gating by DZ and PhB.
from GABA alone in burst duration and burst frequency is
of the opening of chloride channels with multiple conduc-
tance states, but the 26- to 30-pS state was the most
frequent and contributed the majority of current used
in this analysis [16]. At present, it is not clear whether
the multiple conductance states represent different
channels, but since the relative frequency of the states
among patches remained relatively stable, it is likely
that they represent different conformations of the
same channel. In the present study, we showed that
GABA receptor single channel currents were in-
creased by DZ and PhB. Since DZ and PhB did not
alter the conductance of the dominant state, the en-
hancement of GABA receptor chloride current must
have been due to an increase in the mean duration of
channel opening or to an increased frequency of chan-
nel openings or to both.

The present study demonstrated that both DZ and
PhB increased the mean open duration of single
chloride channels evoked by GABA, but that DZ was
more effective. Single channel opening frequency
was not significantly altered. Since records that did not
contain multiple channels were selected for analysis of
single channel properties, the possibility of recruit-
ment of additional channels as a means to achieve en-
hanced whole cell current was not evaluated in this
study.

The single channel results contrast with those deter-
mined by noise analysis techniques. Using noise analy-
sis techniques, Study and Barker [17] compared the
effects of DZ and the effects of the anesthetic barbitu-
rate pentobarbital. DZ increased the “mean channel
lifetime” in 12 of 18 cells, but an increase in frequency
of channel opening was necessary to account fully for
the increase in net current response. Pentobarbital in-
increased “mean channel lifetime” to an extent greater
than that necessary to account for the net current in-
crease and was thus inferred to decrease the “channel
opening frequency.” The discrepancy between noise
estimation and single channel results may be due to
factors involving drug application and concentration
and, more importantly, to the low resolution and lim-
ited frequency bandwidth of noise analysis techniques.

Ligand concentrations generally used in single channel
studies were lower than those used for intracellular
and noise analysis techniques. The concentration of
DZ used in the noise analysis study was 50 to 100
times that used in the present study. No comparable
noise analysis study has been done to compare DZ and
PhB actions on GABA currents, although Barker and
McBurney [18] examined PhB alone at a high un-
known concentration. They found that PhB increased
GABA receptor “mean channel lifetime” to approxi-
mately five times that of control but did not comment
on whether PhB altered the GABA receptor channel
opening frequency. Macdonald and Barker [19] found
that high concentrations of PhB evoked a moderate
amount of chloride current directly, but at the concen-
tration used in this study (500 μM) PhB had little or no
direct effect.

Noise analysis techniques may at times best resolve
average currents with a duration of 5 to 10 msec. Since
single channel recordings have shown that channel
openings are brief and occur in complex bursts, it is
likely that noise analysis techniques may resolve only
bursts of openings. Studies of the ACh receptor chan-
nel have indicated that bursts of openings appear to be
a fundamental response of binding of an agonist to a
receptor-gated ion channel [9]. We examined GABA-
evoked burst properties in the presence of DZ and
PhB. DZ did not prolong GABA-evoked bursts but
increased their frequency. In contrast, GABA-evoked
bursts in the presence of PhB were prolonged but
were not increased in frequency. These burst prop-
ties were similar to those detected by noise analysis for
“mean channel lifetime” and “channel opening fre-
quency.”

The difference between the single channel non-
bursting properties (open time and opening frequency)
and burst properties must be due to the way the channel openings were grouped to form bursts. Single channel studies of the concentration dependence of GABA receptor currents have demonstrated that increasing the concentration of GABA produces (1) an increase in channel opening frequency, (2) an increase in channel mean open duration by shifting the relative proportion of three types of open states to more stable long duration open states, and (3) a shift from isolated brief openings to bursts of long duration openings [20]. Burst prolongation was produced primarily by increasing a population of stable long-duration open states that were incorporated into bursts without altering burst structure or openings. A detailed kinetic analysis of this type is beyond the scope of the present paper; however, DZ appeared to enhance GABA receptor currents primarily by producing a relative shift in the open time population to long-duration states (see Fig 4) and by producing an increase in burst activity without altering bursts. These effects were similar to the effects of increasing GABA concentration.

The effect of DZ may be seen in a hypothetical series of channel openings evoked by GABA (Fig 7A1) and channel openings evoked by GABA in the presence of DZ (Fig 7A2). In this example, GABA evokes openings that occur in isolation and in groups (bursts, indicated by overbar). Openings occurring in bursts tend to be longer than those openings occurring in isolation. In the presence of DZ, GABA receptor bursts increase in frequency and the bursts involve groups of long-duration openings that do not alter the burst duration. In a model for the modulation of bursting activity of the GABA receptor channel, the present results for DZ are consistent with action at the GABA binding site, where DZ is known to increase GABA binding affinity (Fig 7B). Brief, isolated openings that contribute little current are not represented in this model.

PhB, on the other hand, did not produce an increase in the frequency of bursts but potentiated GABA currents primarily by prolongation of bursts. PhB also increased single channel open duration. Burst prolongation without an increase in burst frequency with PhB may have been accomplished by incorporating longer openings and additional openings each time GABA evoked a burst (Fig 7A3). This suggests an action beyond the GABA binding site, perhaps at or near the chloride channel (see Fig 7B). An increase in the number of openings per burst may be caused by a reduction of the likelihood of exiting a burst (intraburst-closed to extraburst-closed transition) or an increased likelihood of reopening once the channel has closed (intraburst closed to open transition) (see Fig 7B).

Thus, modulation of the main conductance state of GABA receptor by DZ and PhB was similar when the general properties of the single channel behavior was examined. Only when the temporal characteristics of the channel were evaluated did details of differential regulation become apparent. It is possible that additional differences concerning burst modulation may be found by further evaluation of burst kinetics.

The patch clamp technique is a powerful method that allows investigation of physiological reactions between receptors and ion channels at a molecular level. Binding assays have demonstrated separate binding sites for DZ and PhB on the GABA receptor complex. The evidence presented here is the first to de-
scribe at the single channel/receptor level two different modes of action for DZ and PhB and suggests that the unique drug binding sites have different regulatory functions at the GABA receptor chloride channel.

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References