The neutral amino acid γ-aminobutyric acid (GABA) produced membrane hyperpolarization and increased membrane chloride ion conductance of spinal cord (SC) and cortical (CTX) neurons in cell culture. GABA dose–response curves were obtained for SC neurons by pressure applying known concentrations of GABA from micropipettes with large tips (miniperfusion pipettes). GABA response threshold was about 2 μM and large responses were elicited at GABA concentrations greater than 10 μM. Bicuculline (BICUC) (0.1–10 μM) reversibly antagonized GABA responses on both SC and CTX neurons with a half maximal inhibitory concentration of about 1 μM. BICUC antagonism of GABA responses was competitive (Lineweaver–Burke analysis). These results are compared with data on GABA and BICUC displacement of [3H]GABA binding to membranes of SC and CTX neurons in cell culture. It is suggested that high affinity GABA receptors are likely to be relevant for postsynaptic GABA responses while low affinity GABA receptors may be presynaptic.

INTRODUCTION

The neutral amino acid γ-aminobutyric acid (GABA) has a widespread distribution in the central nervous system and is a neurotransmitter mediating both pre- and postsynaptic inhibition. GABA interacts with receptors on neuronal membranes to increase chloride ion conductance and thus to displace membrane potential toward the chloride equilibrium potential. Using [3H]-GABA, GABA has been demonstrated to bind to both high and low affinity receptors on brain membranes. The relationship between GABA-binding and GABA-physiology, however, is uncertain. For example, are low or high affinity GABA receptors the physiologically relevant receptors for GABA-mediated postsynaptic inhibition, and which receptors are relevant for GABA-mediated presynaptic inhibition?

Bicuculline is a phthalide-isoquinoline alkaloid which antagonizes GABA-mediated inhibition by binding to the GABA receptors and producing convulsions when administered systemically or applied topically to cortex in animals. Bicuculline has been shown to antagonize GABA responses in central nervous system, in spinal cord, on dorsal root ganglion neurons, on invertebrate neurons, and on muscle. In most preparations where specificity could be studied, bicuculline antagonized only GABA responses and did not alter responses to other amino acids including glycine, β-alanine or glutamate. Bicuculline antagonized GABA responses at concentrations that did not alter either resting membrane potential or membrane conductance. These findings have suggested that bicuculline interacts specifically with GABA receptors.

On spinal cord and cortical neurons in primary dissociated cell culture, GABA also produces inhibition by increasing chloride ion conductance and producing membrane hyperpolarization. In addition, specific antagonism of GABA responses is produced by bicuculline. In a companion paper, we have demonstrated that saturable, sodium-independent binding of [3H]GABA and [3H]-muscimol (a GABA agonist) is present in mem-
branes from mouse neurons in cell culture with binding characteristics similar to those of membranes from adult rat brain. Thus, spinal cord and cortical neurons in cell culture provide preparations in which neurochemical and electrophysiological data can be directly compared.

In this paper we present the dose-dependency of GABA responses and of antagonism of GABA responses by bicuculline. We will compare these results to data presented in a companion paper using [3H]GABA binding and its antagonism by bicuculline to membranes from spinal cord and cortical neurons in cell culture.

MATERIALS AND METHODS

Primary dissociated cell culture

Dissociated neuronal cell cultures were prepared from spinal cords with attached dorsal root ganglia and cortices dissected from 12 to 14-day-old and 15 to 16-day-old mouse embryos respectively as described previously. The cultures were then maintained for 4–12 weeks prior to electrophysiological experiments.

Intracellular recording

Visually guided penetration of spinal cord and cortical neurons was accomplished on the modified stage of an inverted phase-contrast microscope with controlled temperature (35–37 °C). Spinal cord neurons were distinguished from dorsal root ganglion neurons by appearance and identification was confirmed electrophysiologically. Intracellular recordings were made with high impedance (spinal cord neurons, 25–0 MΩ; cortical neurons, 55–70 MΩ) glass micropipettes filled with 4 M potassium acetate (KAc) or 3 M potassium chloride (KCl). Simultaneous current injection and membrane potential measurements with single recording microelectrodes were permitted by using a conventional bridge circuit and data were recorded on a 6-channel Brush polygraph or on film by a Grass camera. Recordings were made from neurons bathed in Dulbecco’s phosphate buffered saline (DPBS; Gibco, Grand Island, NY) with 10 mM Mg 2+ and 1 % glucose. The pH was 7.2–7.4.

Iontophoresis

Iontophoretic application of GABA (Sigma, St. Louis, MO) to neurons during intracellular recording was performed in 10 mM Mg 2+ /DPBS medium which suppressed spontaneous synaptic activity. GABA (0.5 M; pH 1.3 or 3.3) was applied iontophoretically from individual micropipettes (50–100 MΩ) using a constant current stimulator. Brief (50–250 ms) rectangular current pulses (0 to ±34 nA) were used to apply GABA within 2 μm of the neuronal soma membrane preceding, during, and after mini-perfusion of the cell with bicuculline or control medium. Intracellular recordings were made with KCl-containing micropipettes resulting in an increase in intracellular chloride ions and a change of the reversal potential for GABA from ~60 mV to about ~20 mV. Injection of steady constant current through the KCl recording micropipette was used to hyperpolarize the cell membrane to ~70 to ~90 mV and thus GABA responses were depolarizing.

Miniperfusion

Miniperfusion was used to locally superfuse neurons with solutions of known concentration. Miniperfusion was achieved by delivering a pressure pulse to the open shank end (0.9 mm lumen diameter) of a fluid-filled glass micropipette whose tip was large (2–10 μm diameter). A voltage-activated, 3-way pressure valve was controlled by an electronic timer. Air pressure to the valve (1–3.5 lbs/in. 2) was governed by a pressure regulator. When the valve was closed, the miniperfusion pipette was at atmospheric pressure. Pipettes were checked at the beginning of experiments by bringing the pipette tip up against a small neuronal soma in the culture plate on the recording stage of the microscope and observing cell movement during 1–2 s pressure pulses. During intracellular recording, miniperfusion pipettes were positioned 15–100 μm from the soma depending upon the size of their tips. A membrane hyperpolarization, the amplitude of which was pressure-dependent, occurred during fluid ejection with both KAc and KCl recordings for both drug and control solutions. Although this hyperpolarization could be eliminated by decreasing the line pressure, or by moving the pipette further from the soma, a small hyperpolarizing artifact was retained often as assurance of miniperfusion.

Miniperfusion of bicuculline was combined with
Iontophoresis of GABA. The tip of a GABA iontophoresetic pipette was positioned adjacent to a spinal cord neuron after a stable intracellular recording was obtained. GABA was applied at intervals of 2–5 s and iontophoretic current was adjusted to obtain GABA responses of about 10–20 mV. The tip of a bicuculline-containing miniperfusion pipette was then positioned 10-50 μm from the tip of the iontophoresic pipette. If the GABA response was reduced when the bicuculline-containing pipette was positioned (an indication of a ‘leaking’ pipette), the bicuculline-containing pipette was replaced. Care was taken not to direct the miniperfusion pipettes directly at the tip of the amino acid iontophoresic pipettes so the depolarizations produced by these substances were not attenuated by bulk flow from the perfusing pipettes. Control medium or two different concentrations of drug were used in each miniperfusion experiment and dose-dependency was taken as further evidence against a bulk flow effect. Bicuculline concentration was undoubtedly reduced by dilution in the recording medium surrounding the cell during miniperfusion, and therefore the concentration in the pipette was taken as the upper limit of the dose actually at the cell membrane. Long duration (5–15 s) pressure pulses were used.

Bicuculline was dissolved in 0.02 N HCl at the beginning of each experiment and dilutions were made with the DPBS control medium described. The pH of more concentrated bicuculline solutions was adjusted with NaOH when necessary, and all bicuculline solutions were stored at 8 °C in lightproof containers prior to use. All data were obtained within 2 h of preparation of bicuculline-containing solutions at neutral pH since bicuculline is unstable at neutral pH.

Superfusion

Bicuculline was also applied by superfusion of the culture plate. After a stable intracellular recording was obtained, the tip of a GABA iontophoresetic pipette was positioned adjacent to the spinal cord neuron and current was adjusted to achieve a response of about 10–20 mV. Superfusion of control medium at 0.3–0.5 cc/min was then started. If no alteration in GABA response occurred, the culture was superfused by a bicuculline-containing solution and superfusion was continued until the GABA responses were reduced to a stable level (defined as 3 consecutive equal responses). The superfusion medium was then returned to control. With this technique, up to 10 changes with 5 different bicuculline concentrations could be applied to a single cell. Reduction of GABA responses by bicuculline was accepted for analysis only if the response returned to control levels after the bicuculline was replaced by control medium.

Analysis of dose-response data

GABA dose-response curves were obtained by varying the amplitudes of constant duration iontophoresic pulses of GABA. Log-log plots of such dose response data were also made so that the limiting log-log slope at low iontophoresic currents could be measured. The effect of bicuculline on GABA dose-dependency was determined by obtaining control dose–response data and then superfusing the dish with a bicuculline-containing solution. During superfusion, GABA was applied using a constant iontophoresic current. When the GABA response reached a steady reduced value due to bicuculline addition, another GABA dose–response curve was obtained. The bicuculline was then replaced by control medium and dose–response data were again obtained. Data were only accepted if the GABA responses recovered to control values following washout of bicuculline. Lineweaver-Burke plots of the dose–response data were then made to determine the nature of the antagonism of GABA responses by bicuculline.

The dose-dependency of the bicuculline antagonism of GABA responses was studied by obtaining a control GABA response of 10–20 mV amplitude and then superfusing the culture with several different concentrations of bicuculline. Bicuculline was generally added from lowest to highest concentration. Data were accepted only if the GABA responses returned to control values following superfusion with control medium.

RESULTS

GABA hyperpolarized spinal cord and cortical neurons and increased membrane chloride ion conductance

When GABA was applied by miniperfusion to spinal cord (Fig. 1A) and cortical neurons, mem-
brane hyperpolarization and an increase in conductance were produced. Short periods of GABA application (1–2 s) produced responses 10–15 s in duration. If the recording micropipette contained 3 M KCl rather than 4 M KAc, chloride ions entered the neuron changing the equilibrium potential for chloride ion from about —65 mV to —20 mV, and the GABA responses became depolarizing (Fig. 1A2). GABA was also applied to spinal cord and cortical neurons using iontophoresis (Fig. 1B) with 200 ms pulses producing 400–500 ms depolarizing responses (3 M KCl-filled recording micropipettes).

The dose-dependency of GABA responses was studied in spinal cord neurons using miniperfusion of GABA and recording with 3 M KCl-containing micropipettes. In each experiment, 3 miniperfusion pipettes were used, each containing a different concentration of GABA. The cell was impaled by the recording micropipette and hyperpolarized to between —70 and —80 mV. The tip of a single miniperfusion pipette was then brought up to within one cell body (20–40 μm) of the neuron, and GABA was applied for 1 s. The amplitude of the GABA responses recorded using 1–10 μM GABA were averaged and plotted (Fig. 2). Threshold for GABA responses was about 2 μM and large (> 20 mV) responses could be obtained at 10 μM. Log–log plot of the dose–response data (inset Fig. 2) was linear at low GABA concentrations (< 4 μM) but deviated toward the abscissa at higher concentrations. The slope of the linear portion of the log–log plot was 2.8.

**Bicuculline antagonized GABA responses**

Bicuculline rapidly and reversibly reduced the amplitude of iontophoretic GABA responses on spinal cord and cortical neurons (Fig. 3). In spinal cord neurons, bicuculline was applied locally by miniperfusion as well as by bath superfusion, while in cortical neurons only miniperfusion was used. Results obtained using mini-perfusion of bicuculline were similar to those obtained with superfusion of bicuculline if small tipped (10–20 μm) miniperfusion pipettes were used. If larger tips were used, small (10–20%) artifactual reductions of GABA-responses were produced.

Bicuculline had no effect on either resting membrane potential or membrane resistance at the concentrations used in this study (< 10 μM). However, at higher concentrations (> 10 μM), bicuculline produced membrane depolarization with an increase in membrane resistance (not illustrated)19.
Fig. 3. Bicuculline (BICUC) decreased GABA-responses. The scheme on the left shows the experimental paradigm. The data traces at the right show that iontophoresis of GABA evoked depolarizing responses from SC (upper traces) and CTX (lower traces) neurons and that application of 500 nM and 1 μM BICUC decreased the GABA responses. GABA was delivered by 200 ms constant current pulses of 4 nA to SC cells and 34 nA to CTX cells. Membrane potential was increased to −94 mV (SC) and −72 mV (CTX) by passing hyperpolarizing current through the 3 M KCl-filled recording pipette.

Bicuculline reduced the amplitude of GABA responses but did not alter the time course of the responses. The time courses of GABA responses produced by iontophoresis under control conditions and following superfusion of 0.5, 1 and 2 μM bicuculline (Fig. 4) are plotted semilogarithmically. In all cases the GABA responses decayed exponentially with the same time constant (the decay curves are parallel despite each GABA response having a different amplitude).

Antagonism of GABA responses by bicuculline was dose-dependent in both spinal cord and cortical neurons (Fig. 5). In spinal cord neurons (filled triangles), the threshold for antagonism of GABA responses was about 200 nM and 100% antagonism was produced by 10 μM. The IC₅₀ for bicuculline action was about 1 μM. Although we employed fewer bicuculline concentrations with cortical neurons (filled circles), a similar dose-dependency was present. Log–log plots of the data (Fig. 5, inset) were linear at low bicuculline concentrations (< 0.8 μM) but deviated toward the abscissa at higher concentrations. The slope of the linear portion of the log–log dose–response curves was 1.0.

**Antagonism of GABA responses by bicuculline was competitive**

Dose–response curves were obtained for GABA applied by iontophoresis. Such curves were non-hyperbolic, having low response amplitude over the lower GABA iontophoretic charges (current × duration) and steeply increasing response amplitudes at higher iontophoretic charges (Fig. 6). Maximal GABA responses were not achieved and thus responses were only obtained up to 15–20 mV. Log–log plots of GABA dose–response data were linear over low iontophoretic charges but responses deviated toward the current axis at higher charges (Fig. 6).

Fig. 4. BICUC reduced GABA response amplitude without changing response kinetics. GABA response amplitude (mV) from the peak response was plotted as a function of time (ms) during superfusion of 0, 0.5, 1.0 and 2.0 μM BICUC. GABA responses were evoked by 50 ms, 10 nA current pulses. Membrane potential was increased to −78 mV by passing current through the 3 M KCl-filled intracellular recording pipette. Specimen records are presented above the graph.

Fig. 5. BICUC inhibition of GABA responses was dose-dependent. Superfusion and/or miniperfusion of SC and CTX neurons with BICUC reduced the amplitude of GABA responses evoked by iontophoresis. The percent inhibition of GABA responses obtained from 2–13 SC cells and 3–7 CTX cells at each BICUC concentration tested is plotted with BICUC concentration (25 nM–10 μM) on the abscissa. Bars indicate ± S.E.M. Log % decrease of GABA responses vs log BICUC concentration is plotted in inset. GABA was applied by constant current pulses of fixed duration (2.4 nA, 125 ms for data traces above) during application of control and BICUC-containing solutions. Data above were obtained by applying 0, 0.5, 1.0, and 2.0 μM BICUC.
Fig. 6. BICUC altered GABA dose–response curves. GABA dose–response curves were obtained from single SC neurons in control, and in 1 μM BICUC-containing solutions. Medium was changed by superfusion during recording. GABA responses evoked by comparable current pulses were smaller in 1 μM BICUC than in control medium (see data traces) resulting in a shift to the right of the GABA dose–response curve. Log GABA response vs log GABA charge is plotted in the inset. RMP was −57 mV and membrane potential was hyperpolarized to −80 mV by passing current through the 3 M KCl-filled intracellular recording micropipette.

inset). The average slope ± S.E.M. of the log–log plots was 1.34 ± 0.06 (n = 5).

After a dose–response curve was obtained on an individual neuron, bicuculline was superfused into the culture dish, and the dose–response curve was repeated. In the example shown, 1 μM bicuculline shifted the GABA dose–response curve to higher GABA charges (Fig. 6) without changing the log–log slope (Fig. 6, inset). Lineweaver–Burke plots made using reciprocals of response and iontophoretic charge were non-linear and did not intersect the ordinate at positive values. However, Lineweaver–Burke plots made using a power function (n = 2) on the abscissa were linear and intersected the ordinate at positive values (Fig. 7). We used the square of the GABA charge since the log-log slope of iontophoretic GABA dose–response data was about 2.0. Bicuculline altered the intercept on the abscissa without changing the intercept on the ordinate. Furthermore, if we used a non-integer power function on the abscissa to reflect the actual log–log slope of the GABA dose–response curve used in the experiment, bicuculline still altered only the abscissa intercept. Therefore bicuculline appeared to antagonize GABA responses competitively.

DISCUSSION

GABA responses on spinal cord and cortical neurons in cell culture

We have confirmed that GABA produces membrane hyperpolarization of spinal cord2,36 and cortical11 neurons in primary dissociated cell culture by increasing membrane chloride ion conductance. The dose-dependency of GABA responses evoked on spinal cord neurons in cell culture was similar to that reported for cortical neurons in cell culture11 with a threshold of about 1–2 μM. Sodium-independent [3H]GABA binding demonstrated high and low affinity binding sites on membranes from Triton-treated cortical and spinal cord neurons in cell culture18. The potent GABA agonist, [3H]muscimol, bound only to high affinity binding sites using Triton-treated membranes suggesting that high affinity binding sites are the relevant sites for postsynaptic GABA responses. The high affinity GABA binding site had a $K_a$ (9 nM) lower than the threshold concentration of GABA for evoking GABA responses (2 μM) (Fig. 8)15. The difference
Fig. 8. GABA binding occurred at lower agonist concentrations than GABA responses. Sodium-independent high affinity [3H]GABA binding to Triton-treated membranes from spinal cord neurons in cell culture had a lower $K_a$ (9 nM) than the GABA concentration required to evoke threshold GABA-responses (2 μM). For details of binding see Fig. 3 of preceding paper\textsuperscript{15} and Fig. 2 of this paper for details of GABA dose-response curve.

in potency for muscimol and GABA in binding studies and for GABA responses may be due to the removal of an inhibitory protein (or other endogenous modulator) by Triton-treatment\textsuperscript{18,44}.

When a log–log plot of averaged GABA dose–response data was obtained, the curve was linear at low GABA concentrations (1–4 μM) but deviated toward the abscissa at higher concentrations (> 5 μM). The limiting slope of the log–log dose–response curves is an estimate of the Hill number for the agonist receptor interaction\textsuperscript{47}. The log–log slope of the average dose–response curve was greater than one (2.8) suggesting that more than one GABA molecule is required at each GABA receptor to open the GABA-coupled chloride ion channel\textsuperscript{1,2}. The slope of the log–log plot of GABA dose–response data obtained for cortical neurons in cell culture was also greater than one (about two)\textsuperscript{11} suggesting that GABA receptors on cortical and spinal cord neurons are organized similarly.

In contrast, saturation studies of [3H]muscimol binding in Tritonized spinal cord and cortical membranes were hyperbolic and had Hill numbers of one\textsuperscript{15}. Triton treatment of neuronal membranes for binding studies may remove an endogenous inhibitor and alter the kinetics of GABA and muscimol binding to GABA receptors. In addition, receptor desensitization may occur during membrane preparation or during binding experiments where the binding ligand and receptors are in contact for minutes. In the physiological experiments, ligand and receptors are in contact only for seconds. When GABA was applied to spinal cord neurons for longer durations (seconds to minutes), responses to short duration GABA applications (50 ms) became rounded and diminished in amplitude (Macdonald and Barker, unpublished observation). Furthermore, log–log slopes of GABA dose–response curves are reduced to unity. Thus, binding and physiological experiments may not yield equivalent data.

**Bicuculline antagonism of GABA responses**

Bicuculline antagonized GABA responses evoked on cortical and spinal cord neurons at similar concentrations (0.1–10 μM). Bicuculline displacement of [3H]muscimol from high affinity GABA binding sites was produced over a wider concentration range (0.1–1000 μM) but which overlapped the concentration range effective in antagonizing GABA responses\textsuperscript{15}. With spinal cord neurons in cell culture, bicuculline antagonized GABA responses with an IC\textsubscript{50} of about 1.0 μM and displaced specific [3H]-muscimol binding with an IC\textsubscript{50} of about 10 μM (Fig. 9)\textsuperscript{15}. With cortical neurons in cell culture, bicuculline also antagonized GABA responses with an IC\textsubscript{50} of about 1 μM and displaced specific [3H]muscimol binding with an IC\textsubscript{50} of about 5 μM (Fig. 10)\textsuperscript{15}.

Fig. 9. Spinal cord GABA responses were antagonized at BICUC concentrations which displaced [3H]muscimol from spinal cord membranes. BICUC displaced [3H]muscimol between 1 nM and 1 mM BICUC with 50% displacement at about 10 μM BICUC. GABA response amplitude (10–20 mV) was reduced by 0.1–10 μM BICUC with 50% reduction of GABA responses at 1 μM BICUC. For details of displacement experiment see Fig. 5 of preceding paper\textsuperscript{15}. BICUC antagonism of SC GABA responses is from Fig. 5 of this paper.
Fig. 10. Cortical GABA responses were antagonized at BICUC concentrations that displaced [3H]muscimol from cortical membranes. BICUC displaced [3H]muscimol between 1 nM and 1 mM BICUC with 50% displacement at about 5 μM BICUC. GABA response amplitude (10-20 mV) was reduced by 0.1-10 μM BICUC with 50% reduction of GABA responses at 1 μM BICUC. For details of displacement experiments see Fig. 5 of preceding paper15. Details of reduction of cortical GABA responses are presented in Fig. 5 of this paper.

Thus about 10 μM bicuculline will produce a total antagonism of 10-20 mV GABA responses which corresponds to about 50% and 60% inhibition of [3H]muscimol binding to GABA receptors on spinal cord and cortical neurons respectively. The disparity between IC50s of binding and GABA response antagonism probably reflects a difference in the Kd of bicuculline in physiological and binding experimental conditions. In contrast to its inhibition of high affinity [3H]GABA and [3H]muscimol binding, bicuculline displaced low affinity [3H]GABA binding only with high concentrations (Kd = 115 μM)15. The necessity for such high concentrations of bicuculline for blockade of low affinity binding sites suggests that the low affinity site is not responsible for postsynaptic GABA responses. Furthermore, since bicuculline antagonism of high affinity GABA binding and GABA responses occurs at lower and similar concentrations, it is likely that the high affinity GABA binding site is the site relevant for physiological postsynaptic GABA responses.

Multiple GABA-receptors

In addition to binding to high affinity GABA receptors mediating postsynaptic inhibition, GABA has been demonstrated to enhance the specific binding of [3H]benzodiazepines28,43,44. However, there are somewhat different structural requirements for enhancing [3H]diazepam binding and antagonizing [3H]GABA binding suggesting that there are two distinct types of GABA receptors22. Nonetheless, since benzodiazepine receptors and GABA receptors copurify following solubilization17, it is likely that the high affinity GABA receptor and benzodiazepine receptor are part of the same macromolecular complex.

What physiological action is produced by activating low affinity GABA receptors? In addition to producing postsynaptic inhibition, GABA decreases release of neurotransmitter from central and peripheral nervous system neurons4. The pharmacology
of presynaptic GABA receptors, however, is quite different from postsynaptic receptors. Presynaptic GABA receptors are insensitive to blockade by bicuculline, poorly bound by muscimol and potently activated by \(\beta\)-para-chlorophenyl GABA (baclofen). In contrast, postsynaptic GABA receptors are sensitive to bicuculline, potently activated by muscimol and insensitive to baclofen. Low affinity GABA binding sites are not bound by muscimol and are much less sensitive to bicuculline suggesting that low affinity GABA binding sites may be presynaptic GABA receptors.

REFERENCES

2 Barker, J. L. and Ransom, B. R., Amino acid pharmacology of mammalian central neurones grown in tissue culture, *J. Physiol. (Lond.)*, 280 (1978)
23 Krnjevic, K., Chemical nature of synaptic transmission

ACKNOWLEDGEMENTS

We thank Frank Wilk and Robert Frere for technical assistance and Kathy Lundquist for excellent secretarial assistance. This research was supported by NIH Research Grant NS 15225 to R.L.M. and NS 15140 to A.B.Y. L.M.N. was supported in part by NIMH Training Grant 14279 to the Neurosciences Program. A.B.Y. was supported in part by NIH Teacher Investigator Development Award NS 00420. R.L.M. was supported in part by NIH Research Career Development Award NS 00408.


