γ-AMINOBUTYRIC ACID, BICUCULLINE, AND POST-SYNAPTIC BINDING SITES.

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SUMMARY

The binding of γ-aminobutyric acid (GABA) to synaptosomal fractions of the rat cerebellar cortex has been examined at 0-4°C in the presence and absence of bicuculline, chlorpromazine, and/or Na^+. A GABA-binding component has been demonstrated in the synaptosomal fraction which is competitively inhibited by bicuculline. In addition, this binding component persists in the absence of Na^+ and in the presence of chlorpromazine. It seems likely that this binding component is the post-synaptic binding site or "receptor" of GABA.

Considerable evidence exists to suggest that γ-aminobutyric acid (GABA) functions as an inhibitory neurotransmitter in the central nervous system (1-5). In addition, the results of many electrophysiological investigations have demonstrated that the phthalide-isoquinoline alkaloid, bicuculline (BIC), antagonizes the inhibitory action of GABA by competing for the GABA receptor (6-10). Recent investigations have demonstrated that the post-synaptic action of GABA and the uptake or accumulation of GABA are different processes (11). Thus, while iontophoretically applied bicuculline reversibly inhibits the post-synaptic action of GABA (6-10), bicuculline has been reported to have no effect on the GABA transport system (11,12). In addition, while para-chloromercuriphenylsulphonate is a reasonable antagonist of the uptake process, it potentiates the inhibitory action of GABA, presumably by slowing the removal of GABA from the synaptic area while not affecting the post-synaptic GABA receptor (13).

If the post-synaptic receptor and transport mechanisms for GABA involve separate binding sites in the synaptic region, it should be possible to detect the post-synaptic receptor by examining the binding of ^{3}H-GABA

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to synaptosomal fractions, using bicuculline as a specific competitive inhibitor of the receptor. This procedure is analogous to that recently developed in our laboratory for the measurement of the estrogen receptor after translocation to the nuclei of rat uterine cells in which an excess of a competitive inhibitor is employed to determine the amount of specific binding in subcellular fractions (14). In addition, specific binding which is competitively inhibited by bicuculline should be demonstrable in synaptosomes rendered incapable of GABA transport. The transport mechanism for GABA is a chlorpromazine-inhibited, Na^+-dependent process (11,15,16). The purpose of this report is to demonstrate that a GABA-binding component exists in the synaptosomal fraction of the rat cerebellar cortex which binds GABA in a manner which is competitively inhibited by bicuculline and that this binding component persists in the absence of exogenous sodium and in the presence of chlorpromazine (CPZ).

METHODS:

Purdue-Wistar rats (30-45 days old, 75-100 grams) were decapitated, the cerebella quickly excised, and cerebellar cortical tissue hand dissected from the deep nuclei. The tissue was weighed on a torsion balance and homogenized in 10 volumes of 0.32 M sucrose with or without Krebs-Ringer bicarbonate buffer, pH 7.4, at 0-4°C in a Dounce homogenizer using 10 strokes with the A-pestle and 4 strokes with the B-pestle. All procedures were performed at 0-4°C unless otherwise noted. The homogenate was centrifuged at 1000g for 5 minutes. The supernatant was then centrifuged for 20 minutes at 48,000g. The pellet was resuspended in 0.5 ml of the buffer using the previous homogenization procedure, layered on a continuous sucrose gradient (1.5 to 0.7 M), and centrifuged for 60 minutes at 83,000g. The synaptosomes were collected between 1.0-1.15 M sucrose.

Synaptosomes were diluted in either Krebs-Ringer buffer (KR) or a buffer containing 30 mM Tris and 6 mM KCl (TK), pH 7.4, and aliquots equivalent to 10 mg of tissue were added to incubation vials containing various concentrations of ^{3}H-GABA (New England Nuclear Corp., Boston, Mass., specific radioactivity 10.5 Ci/mmole) and 0.2 mM (aminooxy) acetic acid in KR or TK buffer. In some experiments (+CPZ), synaptosomes were pretreated with 0.5 mM chlorpromazine at 0-4°C for 10 min. In addition, bicuculline (0.8 mM) (Pierce Chemical Corp., Rockford, Ill.) was included in the incubation medium as noted in the appropriate figure. After incubation at 0-4°C for 15 min. or 1 hr., the contents of the vials were poured through Millipore filters (pore size, 0.8 μ) (17). ^{3}H-GABA in the

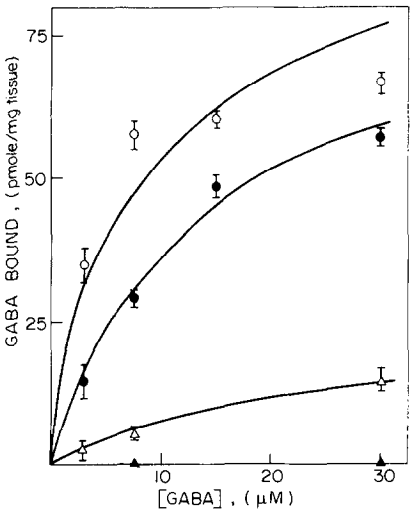

FIGURE 1

Effect of Chlorpromazine and Excess Bicuculline on ^{3}H-GABA Binding and Accumulation. Synaptosomes equivalent to 10 mg of tissue were incubated for 15 min. in KR containing various concentrations of ^{3}H-GABA in the presence (closed symbols) or absence (open symbols) of 0.8 mM BIC. In addition, synaptosomes were preincubated with CPZ (triangles) or had no pretreatment (circles). Means were determined from 3-12 determinations per point.

appropriate incubation medium without the addition of synaptosomes was passed through Millipore filters to serve as a filter blank. The filters were washed with 5.0 ml of the appropriate buffer solution, and placed into scintillation vials containing 1.0 ml of Beckman Bio-Solv. After 15 min. at room temperature, 10 ml of scintillation fluid [99.5% toluene; 0.45% 2,5-diphenyloxazole; 0.05% 1,4-bis-(5-phenyloxazol-2-yl)benzene] was added to the vials and radioactivity was determined with a Packard 3375 liquid scintillation spectrometer. Automatic external standardization was employed to determine the degree of quenching in each sample. The filter blanks were subtracted from all experimental values.

RESULTS AND DISCUSSION:

1. Effect of bicuculline and chlorpromazine on the binding and uptake of ^{3}H-GABA.

Fig. 1 demonstrates the effect of excess (0.8 mM) bicuculline (BIC) and/or 0.5 mM chlorpromazine (CPZ) on the uptake and binding of ^{3}H-GABA by

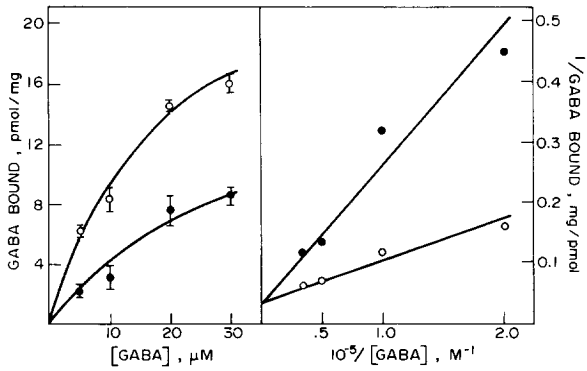

FIGURE 2

Inhibition of ^{3}H-GABA Binding by Bicuculline. Synaptosomes equivalent to 10 mg of tissue were preincubated with 0.5 mM chlorpromazine and subsequently incubated at 0-4°C for 1 hr. with various concentrations of ^{3}H-GABA with or without 100 μM BIC. Each point represents the mean ± S.E.M. of 4 determinations. The apparent K_d for GABA is 21 μM and the apparent K_i for BIC is 80 μM.

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the synaptosomal fraction in a buffer (KR) containing Na^+. In the absence of both CPZ and BIC (open circles), the ^{3}H-GABA retained on Millipore filtration represents that accumulated by transport and that bound to synaptic membranes. Apparent dissociation constants calculated from these curves of mixed function vary between 13 and 20 μM. Bicuculline (0.8 mM) in the absence of CPZ (closed circles) competes with a portion of the ^{3}H-GABA bound in the absence of BIC. The existence of two components, transport and binding, precludes an accurate assessment of dissociation constants or numbers of binding sites in the absence of CPZ. However, in the presence of CPZ (open triangles), the uptake and accumulation of ^{3}H-GABA is remarkably reduced. More importantly, the ^{3}H-GABA bound to the synaptosomal fraction in the presence of CPZ but absence of BIC (open triangles) is competely abolished by the addition of 0.8 mM BIC (closed triangles). CPZ (0.5 mM) abolishes the accumulation of GABA by tissue slices and homogenates (11) while BIC is known to be a competitive inhibitor of the electrophysiologic event (6-10). The present results correlate with these observations (6-10,11) and suggest that the binding sites for GABA which are competitively inhibited by bicuculline in the presence of CPZ (see below) may in fact reside on the post-synaptic receptor site. In these experiments we have employed an excess of BIC in an attempt to ascertain the number of binding sites available which are inhibited by the alkaloid. This level is very high when compared with

Medium and Pretreatment	-BIC (pmol/mg tissue)	+BIC (pmol/mg tissue)	BIC Sensitive* (pmol/mg tissue)
-CPZ, $+Na^+$	66.8 ± 1.6	57.1 ± 1.4	9.7
-CPZ, $-Na^+$	54.5 ± 1.7	45.4 ± 2.5	9.1
+CPZ, $+Na^+$	14.5 ± 2.1	- 0.8 ± 1.6	15.3
+CPZ, $-Na^+$	11.3 ± 1.8	+ 1.9 ± 1.9	9.2

TABLE 1. Effect of Bicuculline, Chlorpromazine, and Na^+ on GABA Binding and Uptake

Aliquots of the synaptosomal fraction equivalent to 10 mg of tissue were incubated for 15 min. at 0°C in Krebs-Ringer ($+Na^+$) or Tris-KCl ($-Na^+$) buffer which contained 30 μM ^{3}H-GABA. In addition, incubation was in the presence or absence of 0.8 mM bicuculline (+BIC and -BIC respectively) with or without pretreatment of the synaptosomes with 0.5 mM chlorpromazine (+CPZ and -CPZ respectively). The values represent the mean ± S.E.M. of 3-6 determinations.

*"BIC-sensitive" is defined as that binding of ^{3}H-GABA in the absence of BIC which is abolished by the presence of excess BIC; that is, (-BIC) - (+BIC).

electrophysiological data but is requisite to competitively inhibit essentially all of the specific GABA sites; that is, a concentration of inhibitor at 10 K_i will inhibit about 91% of the sites while 100 K_i will inhibit 99% of the sites.

2. Competitive inhibition of ^{3}H-GABA binding by bicuculline.

Fig. 2 demonstrates the competitive nature of the inhibition by bicuculline of ^{3}H-GABA binding. Synaptosomes were preincubated with chlorpromazine (0.5 mM) in KR to abolish the transport system and subsequently incubated for 1 hr. with various concentrations of ^{3}H-GABA and bicuculline. The double reciprocal plot reveals an apparent K_d for GABA of 21 μM and an apparent K_i for bicuculline of 80 μM.

3. Na^+ and ^{3}H-GABA binding.

The transport of ^{3}H-GABA is known to be dependent on exogenous Na^+

(15,16). If transport and receptor binding sites are different, it is worthwhile to examine the Na^+ dependency of the GABA binding sites which are inhibited by BIC. Table 1 shows the results of studies in which synaptosomes equivalent to 10 mg of cerebellar cortical tissue were incubated with CPZ, BIC, and/or Na^+. It is apparent that the absence of Na^+ in the incubation medium had no appreciable effect on the "BIC-sensitive" binding of GABA in the absence of CPZ. There may be an effect in those synaptosomes preincubated with CPZ. However, the results of this study suggest that the binding of GABA which is competitively inhibited in BIC is not appreciably affected by exogenous Na^+; that is, the binding of ^3H-GABA which is competitively inhibited by bicuculline is not abolished by the absence of exogenous Na^+.

CONCLUSION:

The persistence of a GABA binding component which is competitively inhibited by bicuculline in the synaptosomal fraction of the rat cerebellar cortex in the presence of chlorpromazine and in the absence of Na^+ suggests that the post-synaptic receptor for GABA can be measured and is different from the transport system. Studies are currently in progress to characterize the GABA binding component which is competitively inhibited by bicuculline and to separate it from the intact synaptosome.

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