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PRESYNAPTIC ACTIONS OF γ-AMINOBUTYRIC ACID AND SOME ANTAGONISTS IN A SLICE PREPARATION OF CUNEATE NUCLEUS

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1 A slice preparation of the rat cuneate nucleus is described which is suitable for electrophysiological studies on the presynaptic action of drugs.

2 Superfusion of a slice with γ-aminobutyric acid (GABA) depolarized the afferent nerves in a concentration-related manner. The responses were Cl^--dependent. Depolarizations to high concentrations of GABA often faded. Glycine and L-glutamate had little effect.

3 (+)-Bicuculline antagonized GABA in an apparently competitive manner ($pA_2 = 5.35$) at low response levels. Strychnine was 10 times less potent. Responses to high concentrations of GABA were sometimes potentiated by (+)-bicuculline and strychnine.

4 Bemegride and leptazol both antagonized GABA, but with low potency and in a manner which was clearly not competitive.

Introduction

Studies on presynaptic inhibition in the cuneate nucleus *in vivo* suggest that this phenomenon is mediated by γ-aminobutyric acid (GABA) receptors on the primary afferent nerve terminals (Banna & Jabbur, 1969; Davidson & Southwick, 1971). Activation of the GABA receptors is thought to be responsible for depolarization of the terminals and a consequent increase in their direct excitability during presynaptic inhibition (Andersen, Eccles, Schmidt & Yokota, 1964). Nevertheless, the pharmacology of presynaptic inhibition (Boyd, Meritt & Gardner, 1966; Banna & Jabbur, 1970) does not always correspond with that of the inhibitory effects of exogenous GABA (see Hill, Simmonds & Straughan, 1976). Some of the discrepancies could be due to the fact that sufficiently detailed pharmacological studies on presynaptic GABA receptors have not proved possible *in vivo*, because of limitations of technique. In the present experiments, therefore, the possibility of using an isolated preparation of the cuneate nucleus has been explored.

The approach adopted was similar to that already used in studies of receptor populations in frog isolated spinal cord (Barker & Nicoll, 1973; Barker, Nicoll & Padjen, 1975; Evans & Watkins, 1975) and rat isolated superior cervical ganglion (Bowery & Brown, 1974; Bowery & Jones, 1976). In these experiments simple recording systems were used to monitor drug-induced changes in membrane polarization, the essential requirement being that the axons of the nerve terminals or cell bodies under study project as a compact bundle for a sufficient distance within the isolated preparation. These studies have also demonstrated the advantages of *in vitro* techniques which permit drugs to be applied at known concentrations and the ionic environment to be varied.

The purpose of the present study, therefore, was two-fold. Firstly, it was necessary to demonstrate that activation of presynaptic GABA receptors in the cuneate slice could be studied quantitatively. This led, secondly, to measurements of the potencies of possible GABA antagonists, selected from the various substances that have been demonstrated to affect presynaptic inhibition. Some of the data have already appeared in a preliminary report (Simmonds & Pickles, 1977).

Methods

Male Wistar rats (250–350 g) were stunned and decapitated at a low cervical level. The medulla oblongata was rapidly exposed, sectioned from the rest of the brain at the level of the obex and placed in ice-cold Krebs medium. The pial membranes were teased completely away and the caudal end trimmed by

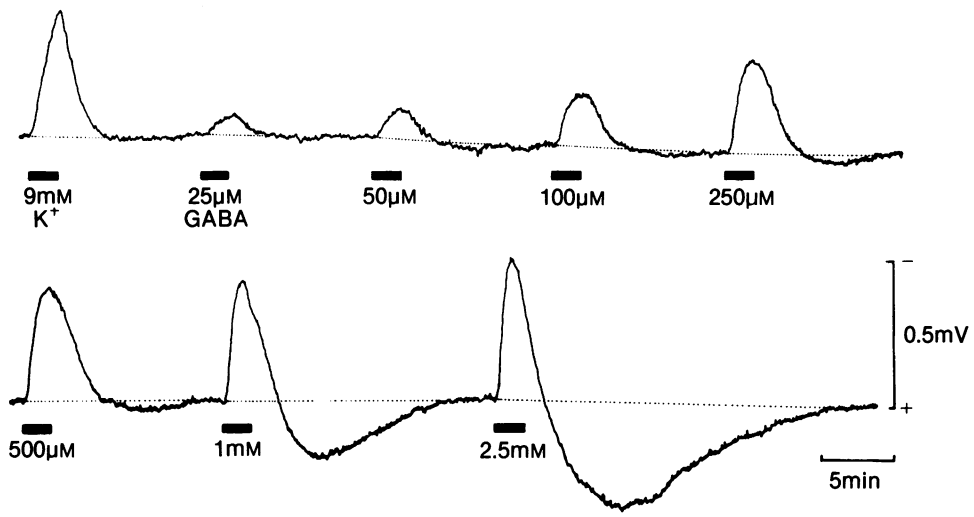

Figure 1 Depolarizing potentials of afferent nerves in the cuneate nucleus slice produced by K^+ (raised to 9 mM from 3 mM) and increasing concentrations of γ-aminobutyric acid (GABA). Contact time was 2 min in each case and the record is continuous. Dotted lines indicate the projected baseline.

making a transverse section 7 mm from the rostral end. The medulla was placed on its ventral surface so that the clearly visible dorsal funiculus on one side of the mid-line was in line with a cutting guide. A razor blade was used to make two vertical sections 500–600 μm apart on either side of the dorsal funiculus. The slice thus produced contained the greater part of the dorsal funiculus on its dorsal margin and the underlying cuneate nucleus.

The caudal end of the dorsal funiculus was then trimmed free of underlying tissue for a distance of 1 to 1.5 mm. All these procedures were carried out with the tissue kept ice-cold. Subsequently, the slice was incubated in Krebs medium at room temperature (20–23°C) for 2.5 to 3 h before being placed in the perfusion chamber.

The arrangement of the tissue in the perfusion chamber was such that most of the slice was in one compartment with just the caudal end of the dorsal funiculus projecting into a second compartment. This was achieved by drawing up the caudal end 1 to 1.5 mm into a nylon suction electrode having a circular orifice of 0.6 mm diameter and containing either Krebs medium or 0.9% w/v NaCl solution (saline); this compartment was not perfused. The main body of the slice was superfused with Krebs medium at 2.0 to 2.5 ml/min at room temperature. Each compartment was contacted by a bridge of 3% agar in saline in which was embedded a Ag/AgCl electrode. The d.c. potential between the two electrodes was recorded differentially on a chart recorder and responses to drugs were measured at their peak amplitude from the projected baseline. The method assumed that a drug-induced negativity in the main slice compartment with respect to the suction electrode indicated a depolarization of the afferent nerve fibres.

The Krebs medium contained (mM) NaCl 118, KCl 2.1, KH_2PO_4 0.93, $CaCl_2$ 2.5, $MgSO_4$ 2.2, $NaHCO_3$ 25 and glucose 11 and was continuously bubbled with 95% O_2 and 5% CO_2. In experiments with 20% of the normal Cl^-, 100 mM NaCl was replaced with Na isethionate. GABA (Sigma), glycine (Koch-Light), L-glutamate (BDH) and KCl were dissolved in the Krebs medium and perfused through the main tissue compartment for 2 min in a dose cycle of 12 to 15 min, or more if the duration of response required it. (+)-Bicuculline (Sigma) was prepared as a 10^{-2} M solution in 0.02 M HCl and added to the Krebs medium just before use; strychnine (Hopkin & Williams), bemegride (Emanuel) and leptazol (pentylenetetrazol, Sigma) were dissolved directly in the Krebs medium. The tissue was superfused with each concentration of these substances for 30 min before and during the application of agonists.

Results

Responses to K^+

Elevation of the K^+ concentration to 9 mM from the 3 mM normally present in the Krebs medium was regularly used as a control depolarizing test. The

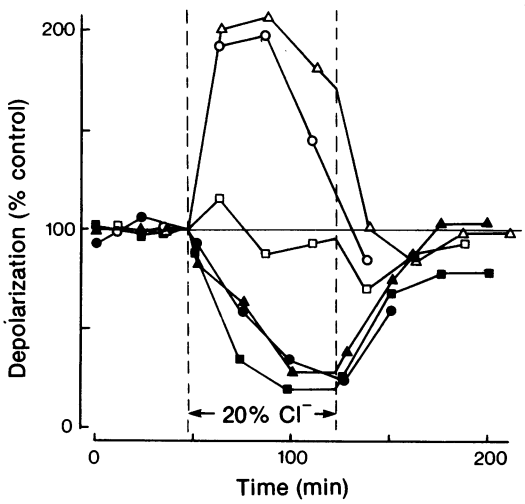

Figure 2 Effect of reducing the Cl^- concentration to 20% of control by replacement with isethionate on the depolarizing responses to K^+ and γ-aminobutyric acid (GABA). The 3 symbols show 3 separate experiments, the open symbols indicating responses to 9 mM K^+ and the filled symbols responses to 50 μM (●, ■) or 100 μM (▲) GABA. Upon changing to low Cl^- medium, there was a substantial shift in the baselines of the original recordings, associated with the establishment of a junction potential. The first GABA dose was, therefore, delayed for 4 minutes.

depolarization increased throughout the 2 min exposure to K^+ (Figure 1) and recovery was occasionally followed by a hyperpolarizing phase before the baseline was re-established. There was no junction potential associated with the increase in K^+, as indicated by the complete loss of response when the dorsal funiculus within the suction electrode was sectioned from the rest of the slice close to the end of the suction electrode.

Responses to GABA, glycine and L-*glutamate*

Superfusion with GABA gave a depolarizing response in each of the 42 slices used in this study. The threshold concentration was about 10^{-5} M and the amplitude of the response was concentration-dependent (Figure 1). At low concentrations, the response was a simple depolarization which usually reached a sustained peak within the 2 min contact time and then recovered to the baseline with little or no subsequent hyperpolarization. At concentrations approaching 10^{-3} M and above, the peak response was often not sustained but began to fall while the GABA was still present. Upon washout, the response sometimes returned slowly to baseline and sometimes reversed rapidly into a rather prolonged hyperpolarizing potential before returning to baseline. In some experiments, the fade of the depolarization in the presence of GABA was sufficiently marked that the maximum of the dose-response curve was not sustained but began to decline with increasing dose. The amplitudes of the depolarizing responses to GABA were reasonably reproducible within an experiment but there was a greater variation between experiments, which generally matched a similar variation in the response to K^+. The cause of the hyperpolarization upon washout of GABA is not known.

To test whether the responses to GABA contained an indirect component involving the release of some endogenous material, the responses were compared in the presence of 20 mM Mg^{2+}. The elevated Mg^{2+} had no effect on responses to GABA.

Glycine and L-glutamate both gave very small depolarizations or no clear response at all. When depolarizations occurred, the threshold concentration was in excess of 10^{-3} M.

Ionic dependence of responses to GABA

The effect of replacing 80% of the Cl^- in the Krebs medium with the less permeant anion, isethionate, was examined. In each of 3 experiments, GABA depolarizations were substantially reduced, with no detectable initial phase of enhancement (Figure 2). However, responses to K^+ were markedly enhanced in 2 out of 3 experiments. Both responses recovered to near control values upon restoration to normal Krebs medium.

Antagonists of GABA

(+)-Bicuculline is now well established as a GABA antagonist (Curtis, Duggan, Felix, Johnston & McLennan, 1971) and it proved effective in the present experiments. The lower part of the GABA dose-response curve was always shifted clearly to the right in a parallel fashion by 1 to 5 μM (+)-bicuculline. However, the responses to high concentrations of GABA, were often facilitated at these concentrations of (+)-bicuculline (Figure 3). This usually occurred when the control responses showed a distinct fade while GABA was still present and at least part of the facilitatory action of (+)-bicuculline appeared to be associated with a reduction of this fade. A similar phenomenon was seen with strychnine at concentrations around 10 μM. No facilitation of responses to low concentrations of GABA was seen.

These data indicated that analyses of drug-receptor interactions which depend on measurements of maximal response could not be applied to these experiments. In subsequent experiments, therefore, only the

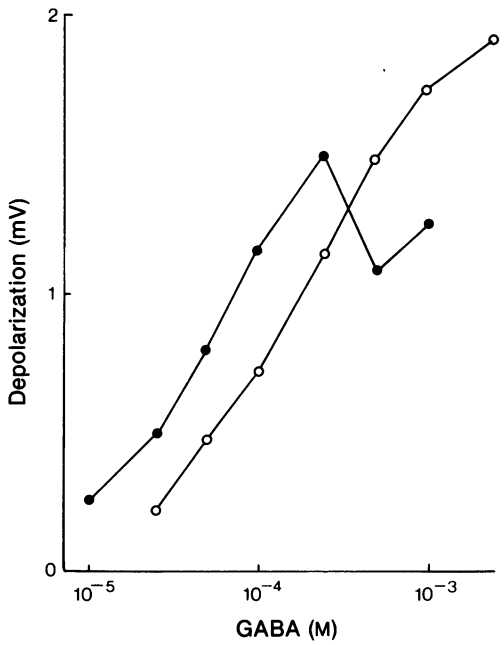

Figure 3 Effect of (+)-bicuculline on depolarizing responses to γ-aminobutyric acid (GABA). Each point represents a single response; filled symbols are control responses and open symbols are responses in the presence of (+)-bicuculline 10^{-6} M. Note that responses to low concentrations of GABA were antagonized but responses to high concentrations were potentiated.

lower part of the GABA dose-response curve was used in a protocol designed to estimate pA_2 and pA_{10} values. Submaximal responses to concentrations of GABA x and $2x$ were established in the control period and applications of $2x$ repeated in the presence of increasing concentrations of antagonist. As the antagonism increased, responses to concentration $10x$ of GABA were also obtained. Thus, in each experiment, the concentrations of antagonist could be determined which were required to reduce the responses to GABA $2x$ and $10x$ to the level obtained with GABA x in the control period.

The effects of four antagonists, (+)-bicuculline, strychnine, bemegride and leptazol are shown in Figure 4 and Table 1. (+)-Bicuculline was the most potent antagonist and the difference (1.10) between the values of pA_2 and pA_{10} was only a little greater than the theoretical 0.95 required to establish competitive antagonism. Strychnine was 10 times less potent and approximated less well to the requirements for competitive antagonism. With bemegride and leptazol, only pA_2 values for GABA antagonism could be obtained. Although both these antagonists shifted GABA dose-response curves in a parallel fashion, the extent of the shift with increasing dose of antagonist was such that the antagonism was clearly not competitive (Figure 5). Consequently, the comparative potencies of these antagonists depended largely on the degree of GABA antagonism at which the comparisons were made.

No attempts were made to follow reversal of antagonism after the high concentrations of antagonist required to determine pA_{10} values. However, responses to K^+ as a control depolarizing agent were well maintained throughout, indicating that there was no general deterioration of the slice preparation. No consistent direct effect of the antagonists on membrane polarization was observed at any stage of the gradual increase in antagonist concentration.

Table 1 Antagonism of γ-aminobutyric acid (GABA)-mediated depolarization of afferent terminals in the cuneate nucleus

	(+)-Bicuculline			*Strychnine*		
	Mean	*Range*	(n)	*Mean*	*Range*	(n)
IC_{50} (μM)	12	3–19	(5)	99	57–120	(4)
pA_2	5.35	4.82–5.95	(5)	4.35	4.12–4.64	(4)
pA_{10}	4.25	4.11–4.52	(4)	2.96	2.70–3.15	(4)
pA_2–pA_{10}	1.10	0.70–1.43	(4)	1.39	1.01–1.94	(4)
	Bemegride			*Leptazol*		
	Mean	*Range*	(n)	*Mean*	*Range*	(n)
IC_{50} (μM)	$>10^3$		(5)	$>10^4$		(4)
pA_2	3.69	3.29–4.20	(5)	2.88	2.60–3.28	(4)

In the control period responses to GABA x were 68 ± 6.2% (mean ± s.d.) of the amplitude of responses to GABA $2x$ (see text).

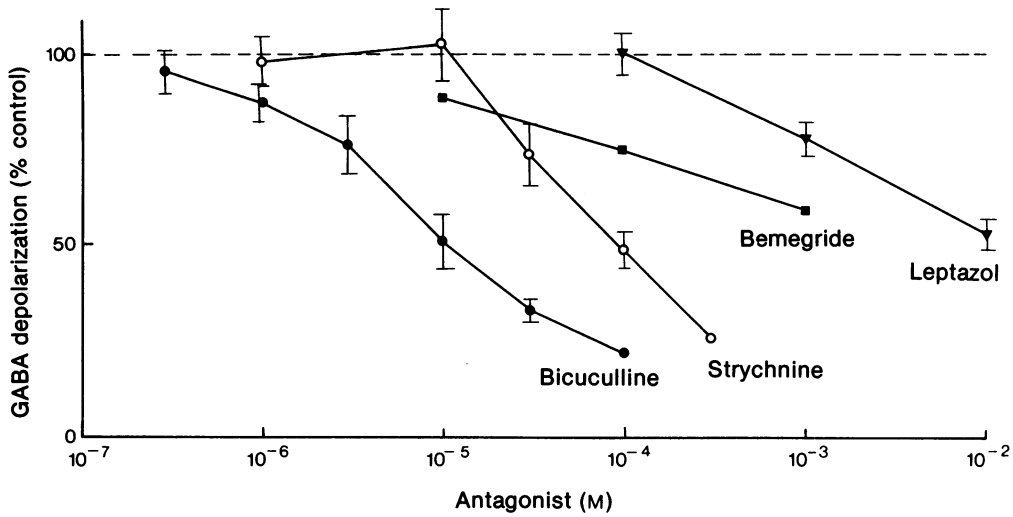

Figure 4 Effects of increasing concentrations of antagonist on the response to a single submaximal concentration of γ-aminobutyric acid (GABA). Each curve shows means from 4 or 5 experiments, vertical lines show s.e. means; where no error bar is apparent, it came within the diameter of the symbol.

Discussion

Of the three amino acids tested on the afferent fibres in the cuneate nucleus, only GABA gave reliable membrane depolarizations. Glycine and L-glutamate had little or no effect. This spectrum of activity accords well with the actions of these amino acids on the dorsal root ganglion cell bodies which give rise to the cuneate primary afferents (De Groat, Lalley & Saum, 1972). On the other hand, both GABA and L-glutamate appear to increase the excitability of primary afferent terminals in the cuneate nucleus (Davidson & Southwick, 1971) and spinal cord (Curtis, Lodge & Brand, 1977) *in vivo*, effects attributed to terminal depolarization. In the cuneate nucleus slice, however, only GABA increases afferent terminal excitability (Hayes & Simmonds, 1978).

It might have been expected from the results of Nishi, Minota & Karczmar (1974), Adams & Brown (1975), Deschenes, Feltz & Lamour (1976), Otsuka & Konishi (1976) and Scholfield (1977) that the GABA responses would be enhanced in low chloride media since the outward electrochemical gradient for Cl^- should be increased. Presumably, this phase occurred in the initial 4 min, before the first dose of GABA was applied, and the subsequent reduction of the GABA responses was due to a substantial loss of internal Cl^-. This might also offer an explanation for the simultaneous enhancement of responses to K^+, if the resting permeability to Cl^- was sufficient to shunt partially the K^+ depolarizations in normal Krebs medium. This possibility is now being investigated.

Since the responses to bath applied GABA were slow and prolonged, there could have been sufficient time for secondary changes in membrane properties and in ion distributions to occur during the period of the primary response to GABA. This might be one mechanism contributing to the fade of the depolarizing responses to high concentrations of GABA, along with the possibility of receptor desensitization. Krnjević, Puil & Werman (1977a, b) have reported a similar fade of the conductance increase and hyperpolarizing response to iontophoretic GABA of spinal motoneurones. These authors also found that bicuculline methochloride reduced the amount of fade, an observation which matches the present findings on cuneate afferents that (+)-bicuculline and strychnine could reduce fade and potentiate responses to high doses of GABA. A similar phenomenon is also apparent in the olfactory cortex slice (H.G. Pickles, personal communication) where the lateral olfactory tract can be depolarized by GABA (Simmonds & Pickles, 1977). Perhaps these observations explain reports that bicuculline and strychnine can sometimes potentiate the inhibitory effect of iontophoretic GABA on neuronal firing (Hill, Simmonds & Straughan, 1973a; 1976) and that strychnine enhances the cuneate P-wave (Banna & Jabbur, 1969).

(+)-Bicuculline, as expected, was an effective GABA antagonist and, on the basis of the pA_2 and pA_{10} values obtained at low response levels, it

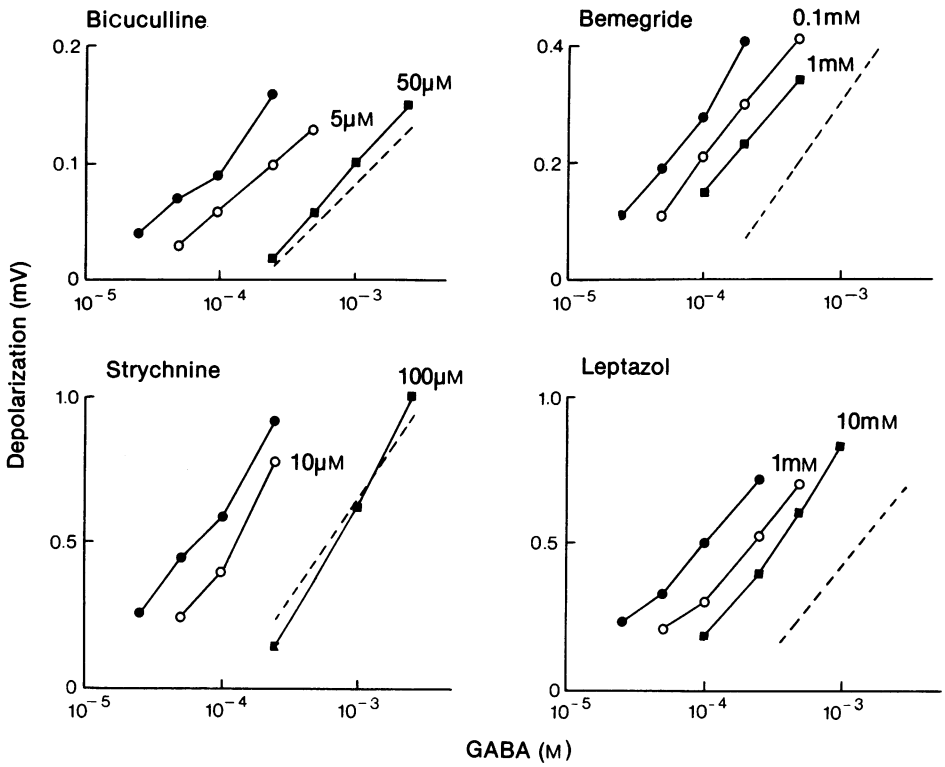

Figure 5 Effects of (+)-bicuculline, strychnine, bemegride and leptazol on γ-aminobutyric acid (GABA) dose-response curves. Each example is a single experiment carried out on a different slice and each point represents a single response. In each case, the control curve (●) was displaced to the right in a parallel fashion by a low concentration of antagonist (○) and further displaced by a ten-fold higher concentration of antagonist (■). If the displacement by the low concentration of antagonist had been competitive, it was calculated that a ten-fold increase in antagonist should have caused a further displacement to the position indicated by the dotted line. In these examples, both (+)-bicuculline and strychnine appeared to be competitive while bemegride and leptazol were clearly not competitive.

appeared to be competitive. However, this could have been fortuitous if the facilitatory effect of (+)-bicuculline extended to low concentrations of GABA and reduced the apparent antagonism of GABA. Such a combination of properties could tend to make an antagonist appear competitive when, in fact, it is not. On the other hand, GABA uptake processes have a proportionately greater effect in limiting responses to low concentrations of GABA (Brown & Galvan, 1977) and this would tend to make a competitive antagonist look less clearly so. Nevertheless, the potency of (+)-bicuculline as a GABA antagonist, whether represented as an equilibrium dissociation constant of 4.5 to 6.3 μM (assuming competitive antagonism) or an IC_{50} of 12 μM, compares quite closely with IC_{50} values of 5 to 7 μM (+)-bicuculline for displacement of [^{3}H]-GABA binding to synaptic membranes from rat brain (Zukin, Young & Snyder, 1974; Enna, Collins & Snyder, 1977) and 14 μM (+)-bicuculline for antagonism of the depolarizing responses to GABA on rat superior cervical ganglion (Bowery & Brown, 1974).

Similar considerations apply to the antagonism of GABA by strychnine. On the basis of the pA_2 and pA_{10} values, an equilibrium dissociation constant for strychnine of 46 to 123 μM (if the antagonism were competitive) or an IC_{50} of 99 μM compares reasonably well with the IC_{50} value of 73 μM strychnine for antagonism of GABA depolarizations in rat superior cervical ganglion (Bowery & Brown, 1974).

The antagonism of GABA by bemegride and leptazol was clearly not competitive so that calculated values of pA_2 can yield no kinetic constants. The concentrations required were high, as also found for leptazol on frog spinal cord (Nicoll & Padjen, 1976), so it is not surprising that no effect was obtained

with microiontophoretic applications *in vivo* (Hill *et al.*, 1976), although iontophoretic leptazol has been reported to antagonize GABA on cultured mouse spinal cord neurones (Macdonald & Barker, 1977).

If antagonism of GABA is to account for the block of presynaptic inhibitory phenomena by bemegride and leptazol (Banna & Jabbur, 1970; Hill, Simmonds & Straughan, 1974a; Hayes, Gartside & Straughan, 1977) as well as by (+)-bicuculline (Banna, Naccache & Jabbur, 1972; Hill, Simmonds & Straughan, 1973b; Hayes *et al.*, 1977), and perhaps their convulsant activity also (Hill, Simmonds & Straughan, 1974b), there should be a reasonable correspondence between the concentrations required for each effect. It can be calculated that the doses required to depress the cuneate P-wave and cause EEG spiking (Hill, 1974), if distributed throughout the extracellular space, would give concentrations of (+)-bicuculline 1 to 2 μM, bemegride 50 to 100 μM and leptazol 500 to 1000 μM. In the present experiments, these concentrations of the three antagonists each caused about a 20% reduction in response to GABA (Figure 4). Strychnine could not be included in the comparison since the concentration of 20 μM required for a 20% antagonism of GABA is far in excess of the concentration required to cause convulsive activity, presumably due to antagonism of glycine. It is possible, therefore, that the ability of (+)-bicuculline, bemegride and leptazol to reduce presynaptic inhibition is due to antagonism of GABA at presynaptic receptors; but the degree of antagonism required is quite small.

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