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CHARACTERIZATION OF AN INHIBITORY RECEPTOR IN RAT HIPPOCAMPUS: A MICROIONTOPHORETIC STUDY USING CONFORMATIONALLY RESTRICTED AMINO ACID ANALOGUES

M. SEGAL[1], K. SIMS[2] & E. SMISSMAN

Laboratory of Neuropharmacology, National Institute of Mental Health, St. Elizabeths Hospital, Washington, D.C. 20032, U.S.A.

1 Pyramidal cells in rat hippocampus were used to study the molecular dimensions of a receptor for inhibitory amino acids in the central nervous system. The inhibitory potencies of γ-aminobutyrate (GABA), β-alanine and glycine were compared by standard microiontophoretic techniques. Subsequently, rigid cyclopentane and cyclohexane amino acid analogues were applied by iontophoresis and their relative efficacies were compared with those of the naturally occurring amino acids.

2 GABA was the most effective of the small aliphatic amino acids in producing inhibition of the firing of hippocampal pyramidal neurones. β-Alanine was less effective and glycine was the least effective. GABA-induced inhibition was antagonized by concurrent iontophoresis of picrotoxin or bicuculline, whereas strychnine did not antagonize GABA inhibition.

3 The ability of the series of substituted aminocyclopentane and aminocyclohexane carboxylic acids to produce inhibition of pyramidal cells was a direct function of the separation of amino and carboxylic acid groups. In both series of the cyclic amino acids the most potent inhibition was demonstrated when the spatial separation was similar to that of the extended GABA molecule (4.74 Å). Additionally, the inhibition of hippocampal pyramidal cells by (±)-*cis*-3-amino-cyclopentanecarboxylic acid, like that produced by GABA, could be blocked by simultaneous application of picrotoxin or bicuculline, but not by strychnine.

4 The present results suggest that the physiologically active conformation of GABA is the fully extended molecule, and additionally indicate that one dimension of the postsynaptic receptor site is within the range of 4.2 to 4.8 ångströms.

Introduction

Previous neurophysiological studies have demonstrated the existence of postsynaptic inhibitory mechanisms in the pyramidal cells of the mammalian hippocampus (Andersen, Eccles & Loyning, 1964). γ-Aminobutyric acid (GABA) has been demonstrated to mimic this postsynaptic inhibition in the hippocampus (Curtis, Felix & McLennan, 1970) and in other test systems within the mammalian central nervous system (CNS) (Obata, Ito, Ochi & Sato, 1967; Galindo, 1969; Curtis, Duggan, Felix & Johnston, 1971; Curtis, Duggan, Felix, Johnston & McLennan, 1971; Bruggencate & Engberg, 1971). β-Alanine and glycine have also been suggested as possible inhibitory neurotransmitters in various CNS systems (Bruggencate & Engberg, 1971) but a detailed comparative study of their effect on the pyramidal cells of the hippocampus specifically has not been reported.

GABA in aqueous solution can exist in numerous molecular conformations, and the spatial separation of amino and carboxy groups can range from 2.4 to 4.8 Å (McGeer, McGeer & McLennan, 1961; Steward, Player, Quilliam, Brown & Pringle, 1971; Van Gelder, 1971; Gilardi, 1973). A previous study using analogues of GABA to characterize the conformation of GABA which is physiologically active on the inhibitory stretch receptor of the crayfish suggests that the optimal spatial separation of the amino and carboxy groups is 4.0 Å or more (McGeer *et al.*, 1961), a separation most consistent with an extended conformation of GABA. Recent studies by Beart, Curtis & Johnston (1971) using a conformationally-restricted analogue, 4-aminotetrolic acid,

[1] Present address: Isotope Dept., Weizmann Institute, Rehovot, Israel.

[2] Present address: Department of Psychiatry, University of Kansas Medical Center, Kansas City, Kansas 66212, U.S.A.

also suggest that the extended form of GABA is the physiologically active conformation. However, studies of the GABA molecule in solid state crystals indicate a spatial separation within the range of 2.7 to 3.1 Å (Steward *et al.*, 1971). Van Gelder (1971), on the basis of theoretical calculations, has suggested that the active conformation is a folded form of the molecule.

The present study represents an attempt to characterize the physiologically active conformation of GABA and thereby indirectly delineate the dimensions of its synaptic receptor site. The present experimental approach utilizes a series of aminocyclohexane carboxylic (ACHC) and aminocyclopentane carboxylic (ACPC) acids. Preliminary findings with the series of cyclohexane amino acids have been reported (Segal, Sims, Maggiora & Smissman, 1973). These results served to illustrate the feasibility of using this experimental approach. However, the incomplete rigidity of the ACHC amino acid did not allow precise definition of the receptor site. For example, several conformers of the same ACHC compound may exist which have markedly different spatial separations of amino and carboxy functions (Table 1). The advantage of the series of ACPC compounds is the rigid and planar cyclopentane ring which holds a constant distance between amino and carboxy functional groups. The ACPC which most exactly mimics the potency of GABA can then be presumed to have the same spatial separation of amino and carboxy groups as in the physiological conformation of GABA; thus, one dimension of the GABA receptor site can be determined. An additional important characteristic of the series of amino acids analogues on which we are currently reporting is that there are no functional groups (e.g., unsaturated bonds or aromatic rings) which would alter the chemical nature of the amino or carboxy moieties.

Methods

Adult (150-250 g) male Zivic Miller rats were used. The rats were initially anaesthetized with 3.5% halothane, tracheotomized and then placed in a stereotaxic apparatus. Anaesthesia was maintained with 0.5-1.0% halothane. A 3 mm diameter hole was trephined in the skull above the dorsal hippocampus (3 mm caudal to the bregma and 2 mm lateral to the midline). The meninges were removed and 3% agar (40° C) in Ringer solution was applied to the exposed cortex. In some experiments the cortex was first removed by suction, then the alveus was exposed and the space was filled with the agar solution. Rectal temperature was monitored continuously and maintained between 36-38° C. Cerebral electrical activity was recorded using the central barrel (3M NaCl, 2-4MΩ, overall tip diameter = 4-6 μm) of a five barrel micropipette, amplified and displayed on an oscilloscope. Extracellular unitary potentials were discriminated from background noise and smaller spikes using a voltage gating device and the voltage output was then integrated at one-second intervals. These one-second values were then plotted as rate of cell firing.

Three of the outer four barrels of the five barrel micropipette were filled with three of the following compounds: 0.5 M GABA (Sigma); 0.5 M β-alanine (Sigma); 0.5 M glycine (Fisher); 10 mM strychnine hydrochloride (Aldrich); 10 mM picrotoxin (Mann); 10 mM bicuculline (Pierce); 1.0 M acetylcholine hydrochloride (Sigma); or one of the aminocyclohexane carboxylic acid or aminocyclopentane carboxylic acid compounds (synthesized by Dr L. Maggiora, University of Kansas, Department of Medicinal Chemistry). The ACPC and the ACHC compounds were dissolved in 0.9% w/v NaCl solution; the racemic compounds were prepared to a concentration of 1.0 M and the non-racemic compounds to 0.5 M. The pH of the resultant solutions ranged between 6.4-7.0 and the compounds were injected as cations.

The fourth outer barrel was filled with 3 M NaCl and used for ejecting neutralizing current (Salmoiraghi & Weight, 1968). A constant current solid-state circuit provided the currents needed to eject or retain the compounds in the barrels, and the circuit automatically neutralized tip currents (Geller & Woodward, 1972).

One of the difficulties inherent in an iontophoretic study is the variability in transport number among different pipettes (Hoffer, Neff & Siggins, 1971). To minimize any effect of pipette variability as a cause of the observed differences among the effects of the various compounds, each compound was tested in at least four micropipettes (Segal & Bloom, 1974). Additionally, the results obtained for each group of compounds were compared statistically using analysis of variance tests.

In testing the relative potency of the individual amino acids, a 10 s pulse (unless otherwise specified) was applied at 1 min intervals. The amount of current needed to produce complete inhibition was first determined and then current was sequentially decreased from a suprathreshold level at each successive pulse until no effect was observed. Two variables were measured, the threshold for a complete blockade of unitary firing and the amount of current needed to reduce spontaneous firing to one-half of the maximal rate. Several of the compounds tested did not produce

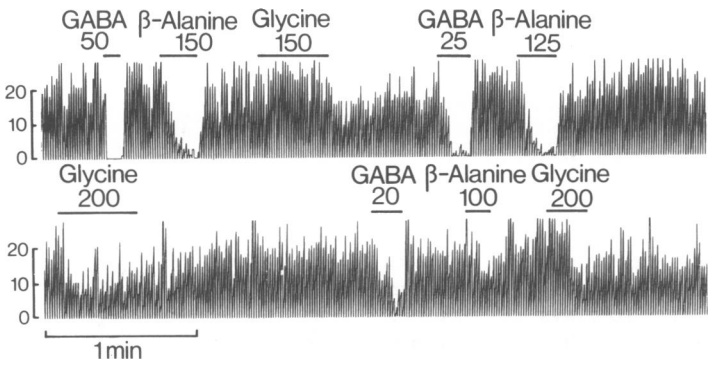

Figure 1 Effects of iontophoretic release of aliphatic amino acids on spontaneous activity of a hippocampal cell. Unit activity was integrated over 1 s intervals. The bar underneath each compound represents the duration of the ejection interval. The numerical value represents the current in nanoamperes (nA) needed to produce the observed change in firing rate. All compounds in this and the following figures were ejected as cations. The ordinate value in this and subsequent figures represents spikes per s intervals.

complete blockade; therefore, current for half-maximal inhibition was chosen as the major variable for comparisons among the compounds.

Results

Cell distribution

A total of 214 cells (15 rats) in the dorsal hippocampus were studied. The majority of the cells was located in a narrow zone of stratum pyramidalea (0.2-0.3 mm ventral to the alveus, 1.9-2.1 mm deep to the surface of the neocortex) of CA1, CA2 and CA3 regions (Lorente de Nó, 1934). Some of the cells were located in area CA3 of the lower blade of dorsal hippocampus (1.2-1.4 mm below the alveus). No differences were observed among the cells in the different regions with respect to sensitivity to the aliphatic and cyclic amino acids tested. Cellular activity was

Table 1 Characteristics of the investigated compounds and summary of the experimental data

	Distance in Å between amino and carbonyl group	*Number of cells*	*Mean current*	σ_x
A. *Natural amino acids*				
Glycine	2.35	20	163.9	20.6
β-Alanine	3.66	21	80.0	7.0
γ-Aminobutyric acid	4.74	65	19.7	1.0
B. *Cyclopentanecarboxylic acids*				
1-amino	2.39	12	221.0	24.2
(±)-*cis*-2-amino	2.54	16	179.3	16.21
(±)-*trans*-2-amino	3.50	10	75.0	6.3
(±)-*cis*-3-amino	4.08	20	18.5	3.1
(±)-*trans*-3-amino	4.77	14	8.9	2.4
C. *Cyclohexane carboxylic acids*				
1-amino	2.39	12	220.8	24.2
(±)-*cis*-2-amino	2.81	30	205.2	15.9
(±)-*trans*-2-amino	2.70*, 3.47**	31	146.3	15.1
(±)-*cis*-3-amino	4.81*, 2.46**	26	94.0	10.6
(±)-*trans*-3-amino	4.04	16	256.2	20.4
(±)-*cis*-4-amino	4.62	10	282.5	17.5
(±)-*trans*-4-amino	5.58*, 4.35**	10	***	***

* equatorial equatorial; ** axial axial; *** largely ineffective.

not recorded from other layers in the hippocampus, (c.f. Herz & Nacimiento, 1965). The cells studied were spontaneously active within a range of 1-20 spikes per second; however, in some cases leakage of acetylcholine was used to increase the firing rate (Krnjević, Mitchell & Szerb, 1963).

Responses to small aliphatic amino acids

The response to GABA was tested in 65 cells. The mean current necessary to produce half-maximal inhibition of firing after 10 s was 19.7 nA (s.e. mean = ± 1.0). The responses exhibited a uniform, characteristic short latency (about 1 s), and frequently, a rebound excitation would follow termination of the iontophoretic current (Figure 1 and Table 1). The response to β-alanine was tested on 21 cells and complete suppression of firing was difficult to demonstrate. The mean current needed to evoke half-maximal inhibitory responses was 80.0 nA, (s.e. mean = ± 7.0). The other small aliphatic amino acid, glycine, was tested on 20 cells. Only a very slight inhibition was observed following glycine iontophoresis and both a large current (163.9 nA, s.e. mean ± 20.6) and a long ejection pulse (up to 1 min) was needed to produce half-maximal inhibition. The difference in mean current required for half-maximal responses among the three amino acids was statistically significant by analysis of variance, ($F_{2,103}$ = 34.86; $P < 0.001$).

Responses to cyclopentane amino acids

The responses to iontophoretic application of (±)-1,3-*trans*-ACPC was determined in 14 cells. This compound was the most potent cyclopentane amino acid inhibitor of spontaneous firing of hippocampal cells among all the compounds tested (Figure 2). The mean current for half-maximum response was 8.9 nA (s.e. mean = ± 2.4) and the inhibition persisted for 5 to 15 s after termination of the ejecting current (Figure 3). The effect of (±)-1,3-*cis*-ACPC was tested on 20 cells. The mean current for half-maximal response was 18.5 ± 3.1 nA and this compound resembled its 1,3-*trans*-counterpart in that inhibition persisted after termination of ejection current (Figure 3), although for a shorter time (2-5 seconds).

The other ACPC compounds were much less effective in inhibiting the firing of hippocampal pyramidal cells (Figure 3). (±)-1,2-*trans*-ACPC (tested on 10 cells) produced half-maximal inhibition at a higher mean ejection current of 75.0 nA (s.e. mean = ± 6.3).

The (±)-1,2-*cis*-ACPC, tested on 16 cells, was even less potent (mean current for half maximal inhibition = 179.3 nA, s.e. mean = ± 16.21). The

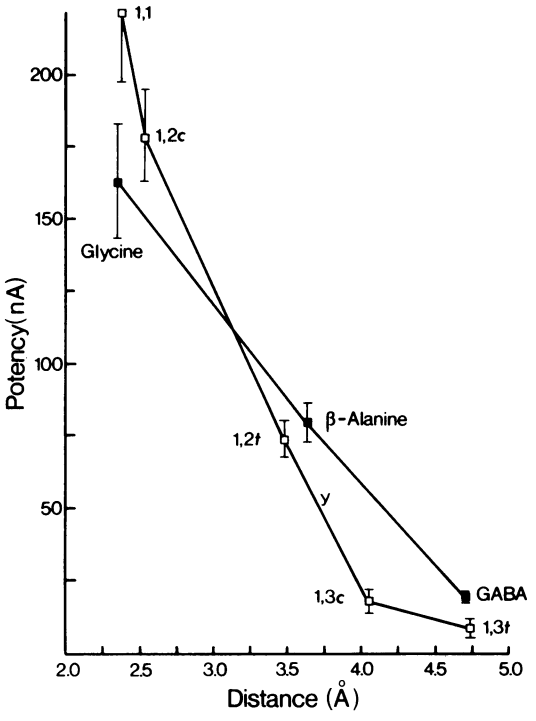

Figure 2 Comparison of the potency of the amino-cyclopentane carboxylic (ACPC) amino acids with those of the small aliphatic amino acids tested. The ordinate values represent current required for half-maximal inhibition. The abscissa value is the distance in ångströms between the amino and carboxy functions (value for the aliphatic compounds represent the value for the fully extended molecule). The results indicate a direct correlation between potency and geometric separation of amino and carboxy groups.

1,1-ACPC was the least effective cyclopentane amino acid and its effect was observed only after large currents (12 cells; mean current for half maximal inhibition = 221 nA, s.e. mean = ± 24.2) and only after the current was sustained for long intervals (Figure 3). The difference among the effects of at least some of the cyclopentane amino acids was highly significant ($F_{4,67}$ = 59.57; $P < 0.001$).

Responses to cyclohexane amino acids

The ACHC compounds were far less active than the ACPC series (Figure 4). The (±)-1,3-*cis*-ACHC derivative was the most effective of the cyclohexane amino acids (Table 1). It produced, in 26 cells, half-maximal inhibitions when ejected with a mean current of 94.0 nA (s.e. mean = ± 10.6).

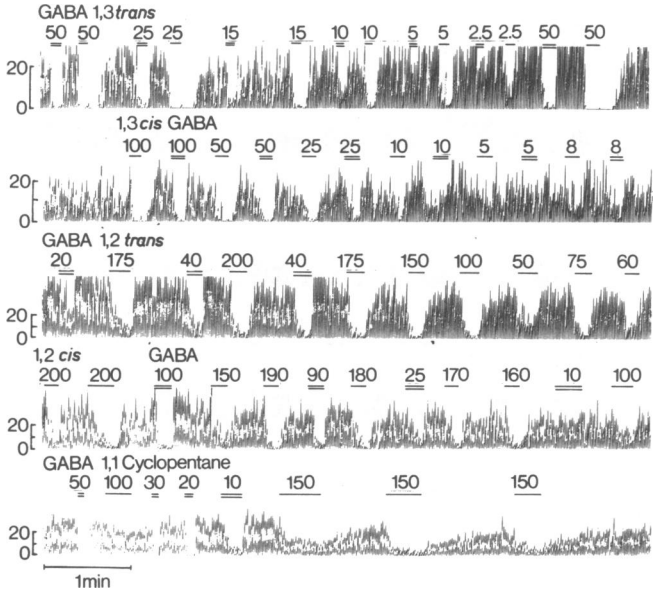

Figure 3 Comparison between the effects of γ-aminobutyric acid (GABA) and the aminocyclopentane carboxylic compounds on firing rates of hippocampal cells. Unitary activity was maintained in these experiments by concurrent continuous application of acetylcholine. No interaction between acetylcholine and the compounds tested on the activity of the cells tested was observed. Note the prolonged inhibitory action of the (±)-1,3-*trans* and 1,3-*cis* cyclic amino acids on the activity of the cells. GABA application is indicated by the double bars delineating the duration of application.

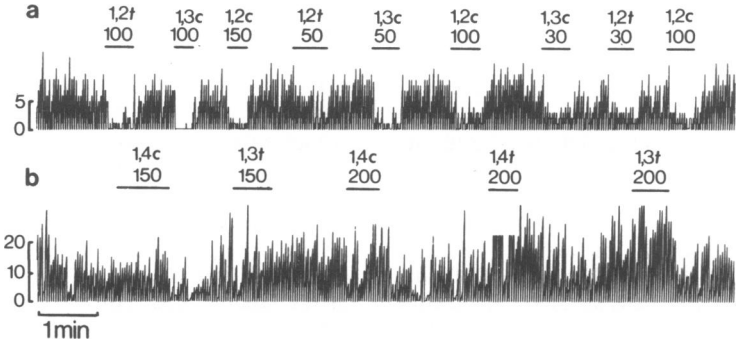

Figure 4 Responses to the aminocyclohexane carboxylic amino acid series. The two traces were taken from two different experiments. Both traces were taken from spontaneously active cells (i.e., no acetylcholine was used to enhance unitary activity).

Next in efficacy was the (±)-1,2-*trans*-ACHC with a mean current of 146.3 nA (s.e. mean = ± 15.3, $n = 31$). For effects to be seen with the other members of the cyclohexane series long ejecting intervals (20-40 s) were required. There was a statistically significant difference in potency among the cyclohexane amino acid ($F_{4,108} = 4.45$; $P < 0.005$).

Antagonism of inhibition

The compounds, bicuculline and picrotoxin (but not strychnine), have been found to specifically antagonize the inhibitory action of GABA in the CNS (Curtis *et al.*, 1971; Barker & Nicoll, 1973). The inhibitory action of (±)-1,3-*cis*-ACPC most closely resembled that of GABA in potency and,

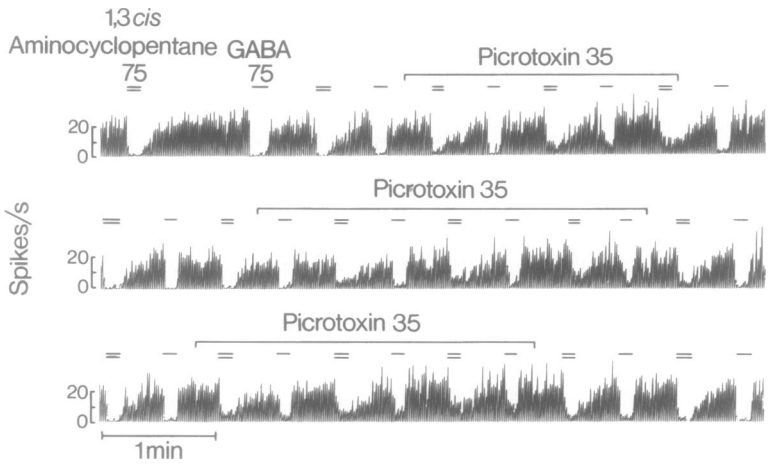

Figure 5 Antagonism of the inhibitory action of γ-aminobutyric acid (GABA) and (±)-1,3-*cis*-aminocyclopentane carboxylic acid by picrotoxin. The three traces are part of a continuous test on one cell and GABA application is indicated by single bars. Note the antagonism of inhibition despite the absence of a direct effect of picrotoxin on cellular activity.

therefore, was utilized as a representative analogue of GABA for comparing the effects of the selective pharmacological blocking agents.

Picrotoxin had a direct excitatory effect on the hippocampal cells tested at large currents (60-100 nA). However, using low currents with no direct effect (10-50 nA), effective antagonism of the inhibitory effect of both GABA and (±)-1,3-*cis*-ACPC was observed. This antagonism was demonstrated (Figure 5) in five out of six cells tested. Bicuculline also produced an increased rate of firing of hippocampal cells when large iontophoretic currents were used. Bicuculline, ejected with low iontophoretic currents which had no direct effect, reduced in an almost identical manner the efficacy of the (±)-1,3-*cis*-ACPC and GABA in three of the five cells studied. Strychnine was tested on six cells. Apart from its direct excitatory action, the agent did not significantly alter the response to either GABA or the (±)-1,3-*cis*-ACPC in any of the four cells tested.

Discussion

The results of the present study indicate that one receptor site mediating inhibition on rat hippocampal pyramidal cells accepts a molecule which has a spatial geometric separation of amino and carboxy functions of 4.6 Å or greater. Among the cyclopentane amino acids, the (±)-1,3-*trans* compound (separation = 4.7 Å) was the most effective in blocking spontaneous activity. Among the cyclohexane amino acids, the 1,3-*cis* compound (separation = 4.81 Å) was the most effective. Of the aliphatic natural amino acids, GABA was clearly the most effective inhibitor of spontaneous firing of hippocampal cells and the fully extended GABA molecule has a separation of carboxy and amino groups of 4.74 Å. These results indicate that the only conformational state of GABA capable of effectively activating the receptor site is the completely (or almost completely) extended molecule. In addition, these data suggest that glycine and β-alanine are not major inhibitory transmitters on hippocampal pyramidal cells. It should be emphasized that these results are applicable only to the cell type tested; i.e., the pyramidal cells of the CA_1 and CA_3 areas of rat hippocampal cortex. It is quite possible that other cell types in the central nervous system that are innervated by GABA-containing terminals may have a different type of receptor which requires a shorter spatial separation of amino and carboxyl functions. This may be the case where both GABA and glycine are postulated as being inhibitory transmitters (Curtis, Hösli, Johnston & Johnston, 1968) and the present approach would provide a means of investigating this interesting phenomenon.

The advantages inherent in the present experimental approach are derived from the use of compounds which: (1) have a well-defined, rigid spatial separation of amino and carboxyl groups; (2) have no other substituent groups which would

significantly alter the chemical reactivity or electronic (charge density) characteristics of the amino or carboxyl groups, and are therefore almost identical to their corresponding aliphatic amino acids; and (3) represent a systematic serial variation of the spatial distance between amino and carboxy moieties. The first advantage is particularly relevant because the precise separation of functional groups for almost all compounds used as pharmacological agents is not known with certainty. Only in recent years, since the advent of the technique of nuclear magnetic resonance spectroscopy, has this facet of pharmacological agents been explored (Sutherland, 1973). The similarity of the cyclic amino acids to the natural amino acids in the electronic characteristics of their functional groups is particularly relevant to studies of drug-receptor relationships. The importance of the charge density of ions acting in a drug-receptor system was recently demonstrated experimentally by New & Richards (1972) using a hapten-antibody model. There, the affinity of antibody was a direct function of the charge density of the ammonium ion in the antigenic site. The similarity in charge density of the cyclohexane and cyclopentane amino acids to their corresponding naturally occurring aliphatic counterparts is illustrated by the almost identical pKa's of the corresponding sets of aliphatic and cyclic amino acids, (Maggiora, unpublished observations). By contrast, the difference in charge density of the carboxy ion which results from introduction of a single acetylenic bond is shown by the wide difference in pKa's of butyric acid ($CH_3-CH_2-CH_2-COOH$, pKa = 4.82) and 2-butynoic acid ($CH_3-C{=}C-COOH$, pKa = 2.65). In this respect, it is pertinent that the compound, 4-aminotetrolic acid, employed by Beart *et al.* (1971), as a 'conformationally-restricted analogue of gamma-aminobutyric acid' would be expected to possess marked chemical differences from GABA.

The present results which indicate that the physiologically active conformation of GABA is the fully extended molecule may provide some insight into the physiochemical basis underlying drug receptor interactions. An aqueous solution of GABA at pH 7.0 consists of a mixed population of conformational states. The geometric separation of amino and carboxy groups may range from 2.4 Å in the 'folded' spatial conformer where the amino and carboxy ions are the most closely apposed to 4.8 Å in the 'fully extended' spatial conformer where the two functional groups are far apart. Previous studies (Segal *et al.*, 1973; Beart, Johnston & Uhr, 1972) using the cyclohexane amino acids indicate that the electrophysiological receptor for GABA may be different from the receptor responsible for GABA uptake. It is then conceivable that the two receptors use different conformational species of GABA. If this is the case, then it is plausible to assume that the physiological effect is terminated not by removal of the physiologically active molecule from the synaptic cleft, but rather by selective removal of a different population of spatial conformers. This is possible because of the extremely rapid equilibrium between the spatial conformations of the same molecule. A practical consideration derived from this concept is that pharmacological blockade of uptake should prolong the physiological effect, but the reverse situation, i.e., blockade of the receptor, should not inhibit uptake. A feasible means of testing this hypothesis of different receptors accepting different spatial conformations of the same molecule would be the use of the cyclopentane amino acid series in experiments testing their ability to affect GABA uptake.

It should be emphasized that this method of using spatial conformers to characterize an amino acid receptor can be extended to other amino acids. Indeed, this basic approach utilizing rigid spatial conformers has been extensively used to characterize acetylcholine muscarinic and nicotinic receptors (Beers & Reich, 1970). Future neurophysiological studies using rigid spatial conformers could add much to our knowledge of the nature and locus of action of neurotransmitters and, at the same time, provide insight into the molecular mechanisms responsible for the observed effect.

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