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THE EFFECT OF CONFORMATIONALLY RESTRICTED AMINO ACID ANALOGUES ON THE FROG SPINAL CORD *in vitro*

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1 The isolated spinal cord of the frog (*Rana pipiens*) was used to examine the structural requirement for the activity of neutral amino acids. The potencies of the aliphatic amino acids, γ-aminobutyric acid (GABA), β-alanine and glycine were compared with the potencies of conformationally restricted cyclopentane and cyclohexane amino acid analogues. Both motoneurone hyperpolarizing and primary afferent depolarizing activity were examined in this study.

2 On motoneurones β-alanine was the most potent aliphatic amino acid and glycine the least potent. Of the substituted aminocyclopentane carboxylic acids, that compound with a separation of amino and carboxylic acid groups closest to that of the extended GABA molecule (4.74 Å) had a potency similar to GABA. As the separation decreased the hyperpolarizing activity fell off rapidly. The substituted aminocyclohexane carboxylic acids were generally inactive even at a concentration of 10 mM.

3 Strychnine blocked the motoneurone hyperpolarizing responses to all compounds with a distance between the amino and carboxylic acid groups of 3.66 Å or less, but did not block the response of compounds with a distance of 4.08 Å or greater. Picrotoxin and bicuculline antagonized all the responses to varying degrees and therefore were of little value in characterizing the responses.

4 On the primary afferents GABA was the most potent aliphatic amino acid and glycine the least potent. The substituted aminocyclohexane carboxylic acids were generally inactive on primary afferents. The response of the substituted aminocyclopentane carboxylic acid whose separation of amino and carboxylic acid groups was closest to that of the extended GABA molecule was most similar to the GABA response. However, (±)-*cis*-3-aminocyclopentane-carboxylic acid (separation = 4.08 Å), which mimicked the action of GABA on motoneurones, closely mimicked the depolarizing action of β-alanine on primary afferents.

5 The findings suggest that the hyperpolarizing GABA receptor on motoneurones will accept a molecule whose amino and carboxylic acid groups are separated by a distance of 4.08 Å or greater while the glycine receptor will accept a compound with a distance of 3.66 Å or less. The depolarizing GABA receptors on primary afferents appear to be more selective since they are not activated by (±)-*cis*-3-aminocyclopentane carboxylic acid (separation = 4.08 Å), while the motoneurone receptors are.

Introduction

The use of a number of convulsant agents which have been found to antagonize the inhibitory effects of amino acids on central neurones (Curtis & Johnston, 1974) has led to the conclusion that most neurones possess two types of receptors for the neutral amino acids: one which accepts short chain amino acids, e.g., glycine, and the other which accepts longer chain amino acids, e.g., γ-aminobutyric acid (GABA) (Curtis & Johnston, 1974). Thus strychnine blocks the action of glycine-like amino acids, while bicuculline and picrotoxin block the GABA-like amino acids. Recent studies on the binding of amino acids and convulsants to brain homogenates provide additional support for this hypothesis (Snyder & Bennett, 1976). On primary afferents the depolarizing responses to the neutral amino acids are somewhat more complex than those reported for central neurones in that strychnine primarily antagonizes amino acids in which the negative and positive charged moieties are separated by two carbon atoms, e.g. β-alanine and taurine (Barker, Nicoll & Padjen, 1975a), there being little effect on the GABA and glycine depolarizations.

Since the major difference in these amino acids is the number of carbon atoms between the amino and carboxyl group, it is generally agreed that it is the distance between the two charged groups that is of

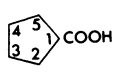
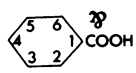
primary importance in conferring the characteristic pharmacological properties on these amino acids. The fact that these amino acids can exist in numerous molecular conformations in aqueous solution have led investigators to use a number of analogues, including conformationally restricted anlogues to determine the conformation of GABA which is physiologically active on a variety of preparations (McGeer, McGeer & McLennan, 1961; Beart, Curtis & Johnston, 1971; Bowery & Brown, 1974; Walker, Azuza, Kerkut & Woodruff, 1975; Segal, Sims & Smissman, 1975; Johnston, Curtis, Beart, Game, McCulloch & Twitchin, 1975; Krogsgaard-Larsen, Johnston, Curtis, Game & McCulloch, 1975; Bowery & Jones, 1976). These investigations have all led to the conclusion that it is the extended form of GABA that is physiologically active. Presumably, as the distance between the amino and carboxyl group is diminished, the amino acid loses its ability to activate GABA receptors and acquires the ability to activate glycine receptors. However, a systematic investigation of this presumption, with a series of conformationally restricted analogues has not been carried out.

The present study compares the effects of a series of aminocyclohexane carboxylic and aminocyclopentane carboxylic acids to the effects of the neutral amino acids, GABA, β-alanine, and glycine in the frog spinal cord. The results obtained on motoneurones have also been compared with the results obtained on pimary afferents to determine if differences exist in the amino acid receptors at these two sites. The use of these analogues on membranes which are considered to have both GABA and glycine receptors should provide insight into the dimensions required for activating these two receptors.

Methods

Techniques similar to those previously described were used (Barker, *et al.*, 1975a). The drug responses were recorded from the spinal roots of the isolated hemisected frog spinal cord with sucrose gap recordings. The drugs were made up in frog Ringer solution and the pH was adjusted when necessary to 7.3 with HCl or NaOH. All experiments were done at a temperature of 9–13°C which was achieved by passing the Ringer solution and drug solution through a thermoelectric cooling unit before entering the sucrose gap chamber. The use of low temperatures increased the size and stability of the motoneurone hyperpolarizing responses. To determine the relative potency of the various compounds to GABA, a concentration of GABA was selected that caused a 20–40% of maximal response. The concentrations of the other compounds were then varied until a response of equal magnitude was obtained (cf. Figure 1). By using concentrations considerably below those causing a maximal response, it was possible to minimize contamination of the motoneurone hyperpolarizing responses by depolarizing components, which require higher concentrations (cf.

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

Compounds	*Distance in Å between amino N and carboxy C atoms*[+]	*Potency relative to GABA* *Monotoneurones (Hyperpolarizing)*	*Primary afferents (Depolarizing)*
A. Natural amino acids	$H_2N(CH_2)_nCOOH$		
Glycine (n = 1)	2.35	0.2 ± 0.08	0.07 ± 0.03
β-Alanine (n = 2)	3.66	2.1 ± 0.5	0.55 ± 0.21
γ-Aminobutyric acid (n = 3)	4.74	1	1
B. Cyclopentanecarboxylic acids			
(±)-*cis*-2-amino	2.54	< 0.002	< 0.005
(±)-*trans*-2-amino	3.50	0.02 ± 0.01	< 0.005
(±)-*cis*-3-amino	4.08	0.37 ± 0.13	0.61 ± 0.17
(±)-*trans*-3-amino	4.77	1.4 ± 0.22	4.25 ± 1.3
C. Cyclohexanecarboxylic acids			
(±)-*cis*-2-amino	2.81	< 0.002	< 0.005
(±)-*trans*-2-amino	2.70*, 3.47**	< 0.002	< 0.005
(±)-*cis*-3-amino	4.81*, 2.46**	0.002	0.02 ± 0.01
(±)-*cis*-4-amino	4.62	< 0.002	< 0.005
(±)-*trans*-4-amino	5.58*, 4.34**	< 0.002	< 0.005

[+] from Segal, *et al.*, 1975, calculated from Dreiding models; * equatorial equatorial; ** axial axial.

Nicoll, Padjen & Barker, 1976). This was particularly true for glycine responses. Magnesium sulphate (10 mM) was added to the Ringer solution to block synaptic transmission in all the experiments except those in which the effect of drugs on root potentials were examined. The following drugs were used in the present study; β-alanine (Aldrich), γ-aminobutyric acid (Aldrich), glycine (Aldrich), glutamate (K & K), bicuculline (K & K). The aminocyclohexane carboxylic acid and aminocyclopentane carboxylic acid compounds were synthesized by Dr L. Maggiora, University of Kansas, Department of Medicinal Chemistry.

Results

Motoneurone response

The relative hyperpolarizing potency of β-alanine, glycine and the active cyclopentane analogues to GABA were determined in 7 preparations and are summarized in Table 1 and Figure 1. For the aliphatic amino acids β-alanine was the most potent and glycine the least. The (±)-1,3-*trans*-cyclopentane analogue was 1.4 times more potent than GABA and the response had a rapid onset and recovery, similar to that of GABA. The (±)-1,3-*cis*-cyclopentane analogue was 0.37 times as potent as GABA and had a slow onset and recovery. The (±)-1,2-*trans*-cyclopentane analogue was 0.012 times as potent as GABA. The (±)-1,2-*cis*-cyclopentane analogue was inactive at a concentration of 10 mM.

The cyclohexane analogues were all inactive at a concentration of 10 mM, except for the (±)-1,3-*cis*-cyclohexane analogue which was 0.002 times as potent as GABA (Table 1).

Primary afferent responses

Similar responses were obtained on the primary afferents except for a few important differences (Figure 2 and Table 1). On the primary afferents GABA was the most potent aliphatic amino acid and glycine the least potent (see also Barker, *et al.*, 1975a). The (±)-1,3-*trans*-cyclopentane analogue was 4.25 times as potent as GABA and had a similar rapid onset and decline of the response, and also exhibited a fade during the application, which is typical of the GABA response. At the low temperature used in these experiments there was usually a distinct hyperpolarizing component to the β-alanine (and glycine) response. This is best seen in Figure 5. The (±)-1,3-*cis*-cyclopentane analogue was 0.61 times as potent as GABA and reached a maximum considerably more slowly than GABA, but similarly to that seen for the β-alanine response. Furthermore, the depolarizing response was invariably followed by a prolonged hyperpolarization. Except for a small hyperpolarization in 2 preparations at a concentration of 10 mM, the (±)-1,2-*trans*-cyclopentane analogue was inactive, as was the (±)-1,2-*cis*-cyclopentane analogue.

The cyclohexane analogues were all inactive at a concentration of 10 mM, except for (±)-1,3-*cis*-cyclohexane analogue which was 0.02 times as potent as GABA (Table 1) and had no hyperpolarizing component.

Effect on root potentials

The compounds which proved to be inactive on motoneurones and primary afferents were also tested

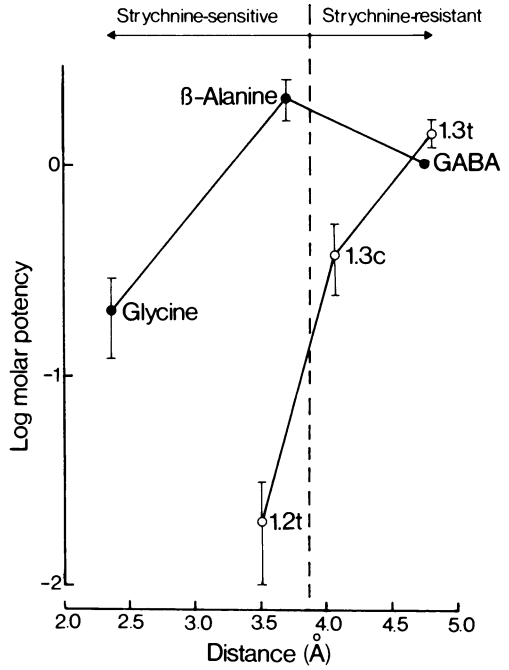


Figure 1 Comparison of the hyperpolarizing potency of aminocyclopentane carboxylic amino acids with aliphatic amino acids of frog motoneurones. The ordinate scale represents the log molar potency relative to γ-aminobutyric acid (GABA) and the abscissa scale is the distance in Å between the amino and carboxy functions; the value for the aliphatic amino acids is for the fully extended molecule. Compounds to the left of the broken line are blocked by strychnine, while those to the right are not affected by concentrations which entirely block the sensitive compounds. The abbreviations are 1,2t, (±)-1,2-*trans*-aminocyclopentane carboxylic acid; 1,3c, (±)-1,3-*cis*-aminocyclopentane carboxylic acid; 1,3t, (±)-1,3-*trans*-aminocyclopentane carboxylic acid.

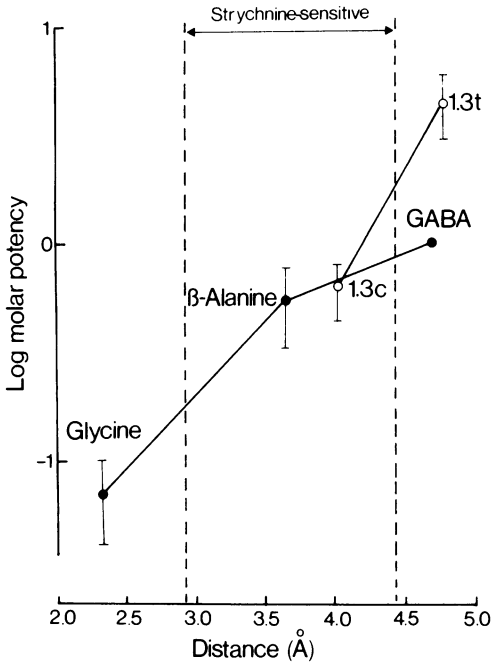


Figure 2 Comparison of the depolarizing potency of aminocyclopentane carboxylic amino acids with aliphatic amino acids on frog primary afferents. The distinction between strychnine-sensitive and strychnine-resistant responses is not as strong as for motoneurone hyperpolarizing responses, since concentrations of strychnine which completely block β-alanine responses also reduce to some extent the (±)-1,3-*trans*-aminocyclopentane carboxylic acid response. See legend to Figure 1 for further details and abbreviations.

on dorsal root potentials and ventral root potentials. It is possible that these compounds might affect the excitability of interneurones, if the receptors on these cells differed from those examined above. If this were the case then one would expect a depression of the polysynaptic ventral root potential (cf. Curtis, Phillis & Watkins, 1961). In addition, if these compounds were able to block amino acid receptors or block amino acid uptake, one might expect an antagonism of dorsal root potentials in the former case or a prolongation of these potentials in the latter case, since neutral amino acids are thought to mediate dorsal root potentials (Barker & Nicoll, 1972; Davidoff, 1972; Barker *et al.*, 1975a,b). The following cyclohexane analogues were examined at a concentration of 5 mM; (±)-1,2-*cis*, (±)-1,2-*trans*, (±)-1,4-*cis* and (±)-1,4-*trans*. All of these compounds were found to have little effect on either the dorsal or ventral root potentials.

Effects of antagonists on motoneurone responses

Concentrations of strychnine which antagonized the hyperpolarizing responses to glycine and β-alanine also antagonized the hyperpolarizing response of the (±)-1,2-*trans*-cyclopentane analogue (Figure 3a). There was no effect on the (±)-1,3-*trans*-cyclopentane analogue with concentrations of strychnine which completely blocked the above responses. The (±)-1,3-*cis*-cyclopentane analogue response was generally not affected by strychnine, although the response in Figure 3a was slightly reduced by strychnine.

In three preparations with particularly good hyperpolarizations to β-alanine, the action of strychnine on dose-response curves was examined. However, the results were difficult to interpret because of the underlying depolarizing component to the β-alanine response, which is more prominent with high concentrations and is less sensitive to strychnine than the hyperpolarizing component (Nicoll, *et al.*, 1976).

The results obtained with picrotoxin and bicuculline were of little value in characterizing the amino acid responses, because their actions were so variable and, in general, nonspecific (cf. Nicoll, *et al.*, 1976). In Figure 4a picrotoxin antagonized all of the hyperpolarizations with a somewhat greater effect on the β-alanine and the (±)-1,3-*cis*-cyclopentane analogue response. In 2 other preparations the β-alanine responses was slightly less sensitive to picrotoxin than the other 3 responses. Bicuculline proved to be equally nonspecific in its action (Figure 4b).

Effects of antagonists on primary afferent responses

Picrotoxin antagonized the depolarizing responses to β-alanine and the (±)-1,3-*cis*-cyclopentane analogue to the same extent (Figure 5). If there were a hyperpolarizing component to the β-alanine response it was less sensitive to picrotoxin, but could be blocked with high concentrations (0.4 mM). On the other hand, the hyperpolarizing response to the (±)-1,3-*cis*-cyclopentane analogue was quite resistant to the action of picrotoxin. The sensitivity of the (±)-1,3-*trans*-cyclopentane analogue to picrotoxin lay between that of GABA on the one hand and β-alanine and (±)-1,3-*cis*-cyclopentane analogue on the other (Figure 5).

The depolarizing responses to the (±)-1,3-*cis*-cyclopentane analogue had a similar sensitivity to strychnine as did the β-alanine response (Figure 3b). However, the hyperpolarizing component of the β-alanine (and glycine) response was blocked by strychnine, while the hyperpolarizing response to (±)-1,3-*cis*-cyclopentane analogue was entirely resistant. The action of strychnine on the (±)-1,3-*trans*-cyclopentane response was quite variable, but in all 5 preparations, some degree of antagonism was present. However, it was considerably less sensitive to

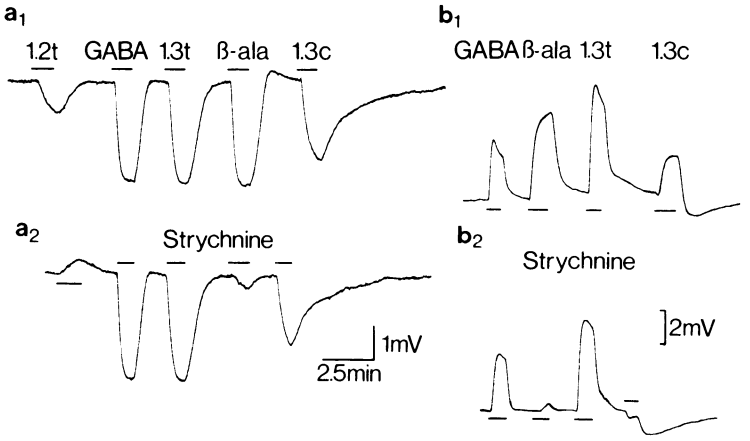


Figure 3 Effect of strychnine on motoneurone and primary afferent responses: (a1) shows the control ventral root responses to 1,2-*trans*-aminocyclopentane carboxylic acid (1,2t, 5 mM), γ-aminobutyric acid (GABA, 0.4 mM), 1,3-*trans*-aminocyclopentane carboxylic acid (1,3t, 50 μM), β-alanine (β-ala 0.4 mM), and 1,3-*cis*-aminocyclopentane carboxylic acid (1,3c, 0.5 mM). The record in (a2) was begun 15 min after changing to a Ringer solution containing 1 μM strychnine. (b1) Shows the control dorsal root responses to GABA (0.5 mM), β-alanine (β-ala, 1 mM), 1,3t (0.5 mM) and 1,3c (1 mM). The record in (b2) was begun 20 min after starting strychnine (20 μM). The time calibration in (a) also applies to (b).

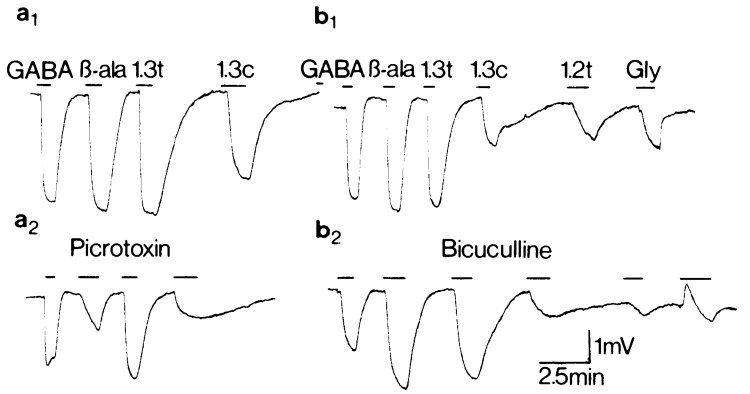


Figure 4 Effect of picrotoxin and bicuculline on motoneurone responses: (a1) shows the control responses to γ-aminobutyric acid (GABA, 0.2 mM), β-alanine (β-ala, 0.1 mM), 1,3-*trans*-aminocyclopentane carboxylic acid (1,3t, 0.4 mM) and 1,3-*cis*-aminocyclopentane carboxylic acid (1,3c, 1 mM). The responses in (a2) were obtained 10 min after starting a Ringer solution containing 5 μM picrotoxin. The control responses in (b1) were obtained with the following concentrations GABA (0.2 mM), β-alanine (β-ala, 0.2 mM), 1,3t (0.2 mM), 1,2t (5 mM) and glycine (Gly, 0.2 mM). The responses in (b2) were obtained 15 min after changing to a Ringer solution containing 50 μM bicuculline. The calibration in (b) also applies to (a).

strychnine than were the β-alanine and the (±)-1,3-*cis*-cyclopentane analogue responses. At the low temperatures used in these experiments, concentrations of strychnine which completely blocked the β-alanine response often resulted in a small decrease in the GABA response (10–15%) which did not appear to be due to a gradual deterioration of the preparation.

Discussion

In the present study an attempt was made to determine the conformational requirements of a compound for activating glycine and GABA receptors in the frog spinal cord. The advantages derived from using aminocyclohexane carboxylic acids and especially aminocyclopentane carboxylic acids in such

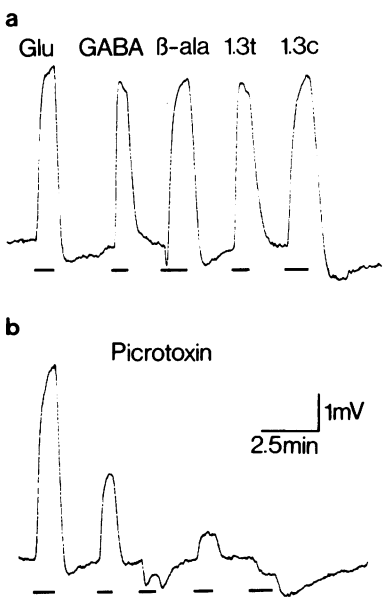


Figure 5 Effect of picrotoxin on primary afferent responses. The control responses in (a) were obtained with the following concentrations: glutamate (Glu, 1 mM), γ-aminobutryic acid (GABA, 0.4 mM), β-alanine (β-ala, 1 mM), 1,3-*trans*-aminocyclopentane carboxylic acid (1,3t, 80 μM), and 1,3-*cis*-amino-cyclopentane-carboxylic acid (1,3c, 1 mM). The responses in (b) were obtained 15 min after starting a Ringer solution containing 0.1 mM picrotoxin.

a study have been discussed (Segal *et al.*, 1975). It should be kept in mind that the dual action of many of the compounds may have introduced small errors in relative potency, but this problem was minimized by appropriate adjustment of amino acid concentrations. It is also possible that endogenous amino acids may be released from storage sites by structural analogues, as has been shown in the sympathetic ganglion (Bowery, Brown, Collins, Galvan, Marsh & Yamini, 1976). The results with the cyclopentane analogue series on motoneurones proved to be the most informative of this study and indicate that those molecules which have a spatial geometric separation of amino and carboxy groups closest to that of the fully extended GABA molecule (separation = 4.74 Å) are most effective in activating GABA receptors. The present findings are qualitatively similar to those of Segal *et al.* (1975) on hippocampal neurones and to other studies with other analogues on a variety of preparations (McGeer, *et al.*, 1961; Beart, *et al.*, 1971; Bowery & Brown, 1974; Walker, *et al.*, 1975; Krogsgaard-Larsen, Johnston, Curtis, Game & McCulloch, 1975; Johnston, *et al.*, 1975; Bowery & Jones, 1976). However, as the distance between the amino and carboxy groups was decreased for the cyclopentane analogues, the potency fell off much more rapidly than in hippocampal neurones, which is surprising in that frog motoneurones are more responsive to shorter chain amino acids than are hippocampal neurones. This might be explained by steric effects that can be expected with substitution of ring structures between the amino and carboxy group which would alter receptor preference independent of the distance between the amino and carboxy group.

Of the amino acid antagonists used in this study, strychnine was the most selective in its action on motoneurones and provided a means for determining whether the analogues activated GABA or glycine receptors. (Although β-alanine was the most potent of the strychnine-sensitive amino acids, the receptor involved in these responses will be referred to as the glycine receptor, in keeping with terminology in the mammalian CNS (Curtis & Johnston, 1974)). All of the active compounds with a distance between the amino and carboxy group of 3.66 Å (as in β-alanine) or less were blocked by strychnine, while those with a distance of 4.08 Å (1,3-*cis*-cyclopentane analogue) or greater were unaffected by concentrations of strychnine which entirely blocked the sensitive responses. These results would imply that on frog motoneurones a very sharp cut-off point exists for compounds which activate GABA receptors and for those that activate glycine receptors. It was hoped that picrotoxin and bicuculline would provide complementary evidence for the findings obtained with strychnine, since Segal *et al.* (1975) reported that these convulsants antagonize the action of the 1,3-*cis*-cyclopentane analogue. However, picrotoxin and bicuculline proved to be of little use in distinguishing between the two receptors, since all of the responses were affected to varying degrees. This nonselectivity on frog motoneurones (cf. Nicoll *et al.*, 1976) is in contrast to results obtained with neurones in the cat CNS (Cf. Curtis & Johnston, 1974).

The results obtained from primary afferents differed considerably from those on motoneurones and are somewhat more difficult to characterize. As seen with motoneurones the cyclopentane analogue (i.e., 1,3-*trans*), with dimensions closest to that of the fully extended GABA molecule, most closely mimicked the response to GABA except that strychnine antagonized the response to some extent. Unlike the findings on motoneurones the depolarizing response to the (±)-1,3-*cis*-cyclopentane analogue had properties similar to the β-alanine response rather than the GABA response. The time course, potency and sensitivity to strychnine and picrotoxin of the depolarizations to these two compounds were remarkably similar. However, the hyperpolarizing component to the β-alanine (and glycine) response was blocked by strychnine whereas that elicited by the 1,3-*cis*

compound was not. In addition, as reported earlier (Barker *et al.*, 1975a), the depolarizing response to glycine was not blocked by strychnine.

It is not clear why most of the cyclohexane analogues which have distances between their amino and carboxy groups comparable to those for the aliphatic amino acids in their fully extended form and to the cyclopentane analogues showed little activity. Interestingly, Segal *et al.* (1975) found the cyclohexane analogues to be weakly active on hippocampal neurones. Furthermore, these compounds had little effect on root potentials which are thought to involve the release of neutral amino acids (Barker & Nicoll, 1972; Davidoff, 1972; Barker *et al.*, 1975a, b) suggesting that they neither compete for neutral amino acid receptors nor uptake sites.

In summary, the major difference between the hyperpolarizing motoneurone responses and the primary afferent depolarizing responses is that the strychnine sensitive receptors on motoneurones will accept a compound with a distance between the amino and carboxy functions of 3.66 Å or less, whereas the strychnine senstive depolarizing receptors on primary afferents will accept a compound with a distance larger than 2.35 Å (glycine) and smaller than 4.74 Å (GABA). However, the selectivity of strychnine is not as great as on primary afferents since the response to the (±)-1,3-*trans*-cyclopentane analogue (separation = 4.77 Å) is affected to some extent.

I wish to thank Dr L. Maggiora for synthesizing the compounds used in the present study and Dr M. Segal for his gift of (±)-1,3-*trans*-cyclopentane and (±)-1,2-*cis*-cyclopentane. The critical reading of the manuscript by Dr A. Trevor is appreciated. This work was supported by a PMAF Starter Grant and a grant from the Academic Senate of the University of California, San Francisco.

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