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Depolarizing responses to glycine, β-alanine and muscimol in isolated optic nerve and cuneate nucleus

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1 Concentration-dependent depolarizations were evoked by glycine and β-alanine 5×10^{-4} – 10^{-2}M and by the γ-aminobutyric acid (GABA) analogue, muscimol 10^{-6} – 10^{-4}M.

2 The maximal response to glycine was several-fold higher than that to muscimol on optic nerve but the reverse was found on the dorsal funiculus fibres in the cuneate nucleus. β-Alanine evoked a similar maximal response to glycine on optic nerve but a considerably higher maximum than glycine in the cuneate nucleus.

3 Strychnine was 19.5 times more potent as a glycine antagonist ($pA_2 = 6.58$) than as a muscimol antagonist. Bicuculline was 156 times more potent as a muscimol antagonist than as a glycine antagonist. Other antagonists of muscimol, i.e. tubocurarine, picrotoxin and leptazol, and potentiators of muscimol, i.e. pentobarbitone and flurazepam, had little or no effect on responses to glycine.

4 Responses to β-alanine had pharmacological properties compatible with a mixed action on both GABA and glycine receptors.

5 The rat isolated optic nerve appears to be a useful preparation for studying the pharmacology of the neuronal glycine receptor plus chloride ionophore complex.

Introduction

There is ample evidence that responses to glycine are distinct from those to γ-aminobutyric acid (GABA) in the mammalian central nervous system although both are inhibitory and involve an increase in chloride conductance (Krnjević, 1974). The main distinguishing feature is that responses to glycine are much more readily blocked by strychnine than are responses to GABA (Curtis, Duggan, Felix, Johnston & McLennan, 1971; Hill, Simmonds & Straughan, 1976). The potencies and sites of action of several GABA antagonists have been determined on isolated preparations of rat cuneate nucleus (Simmonds, 1982) but comparable quantitative information for glycine was not obtained from this preparation due to the small size of the glycine responses.

In the search for an isolated preparation from the central nervous system which responds well to glycine, the rat optic nerve appeared promising. Therefore, this preparation was compared with the cuneate nucleus regarding responses to glycine, β-alanine and muscimol. The potencies and selectivities of antagonists and potentiating drugs were also determined.

Methods

Male Wistar rats were stunned and decapitated. A 3–4 mm length of each optic nerve was removed from within the skull and placed in Krebs medium at room temperature. Alternatively or additionally, the medulla oblongata was removed and slices containing the dorsal funiculus and cuneate nucleus were prepared as previously described (Simmonds, 1978, 1980). The optic nerves and cuneate nucleus slices were placed in similar two-compartment baths so that the nerve or dorsal funiculus, respectively, projected through a greased slot in the barrier between the compartments. Both compartments were perfused with Krebs medium at room temperature with the tissues submerged. Drug solutions diluted in Krebs medium were perfused through one compartment only. The potential difference between the two compartments was recorded continuously and negativity induced in the drug-containing compartment was interpreted as a depolarization of the projecting nerve fibres in that compartment. The responses were measured at their peak amplitudes.

Muscimol (Fluka) was used as the agonist at GABA receptors for comparisons with glycine

(Koch-Light) and β-alanine (Sigma). These and other prospective agonists were superfused for periods of 2 min. Prospective antagonists and potentiators were superfused for 30 min before and during the re-determination of agonist responses. (+)-Bicuculline (Sigma) was prepared as a 10^{-2} M solution in 0.02 M HCl and added to the Krebs medium just before use. Picrotoxin (Sigma), strychnine (Hopkin & Williams), pentobarbitone sodium (Sigma) and flurazepam (Roche) were dissolved directly in the Krebs medium.

The Krebs medium contained (mM): NaCl 118, KCl 2.1, KH_2PO_4 1.2, $CaCl_2$ 2.5, $MgSO_4$ 2.2, $NaHCO_3$ 25 and glucose 11 and was continuously gassed with 95% O_2 plus 5% CO_2. In some experiments, part of the NaCl was replaced with an equimolar amount of Na isethionate to produce a medium with 20% of the usual chloride concentration.

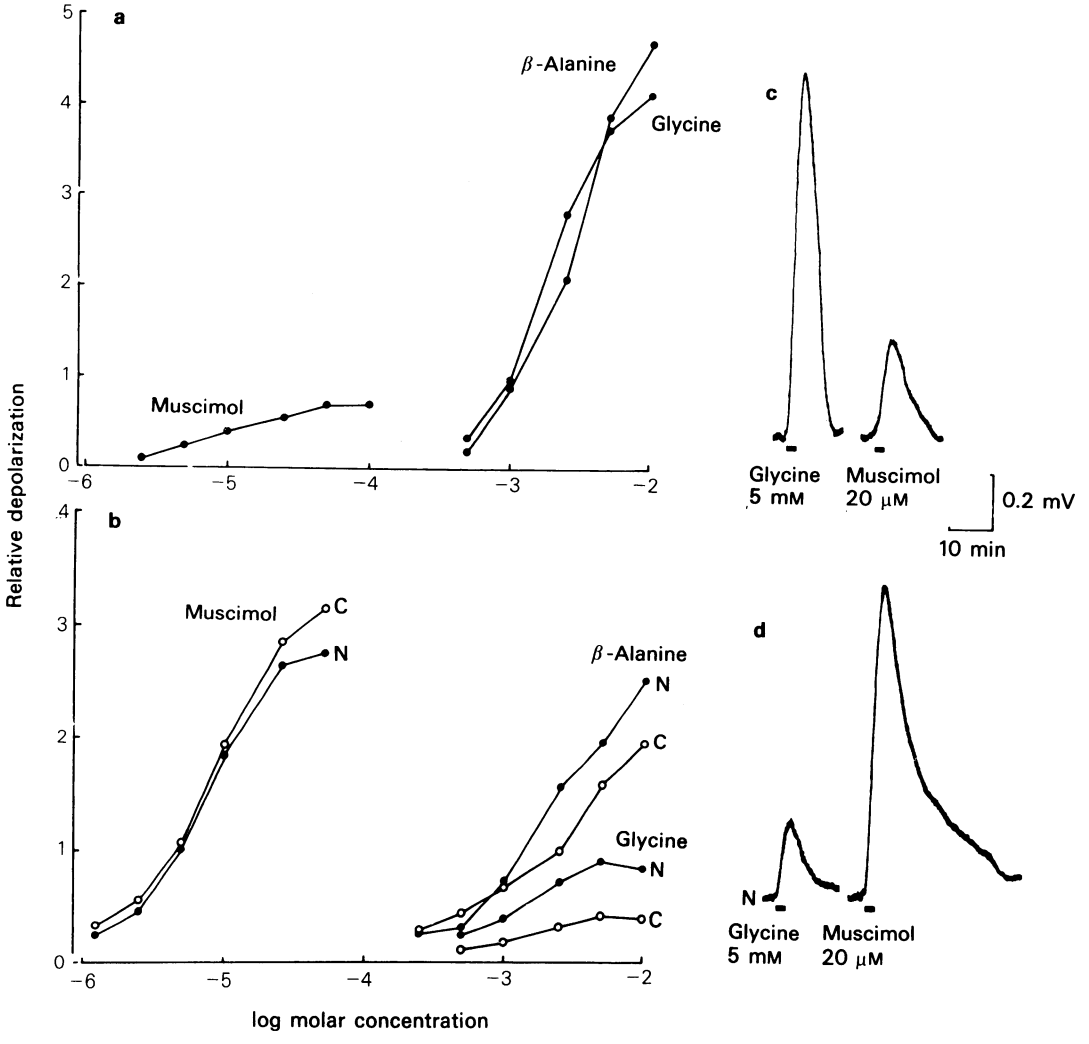

Figure 1 Responses of the optic nerve (a) and cuneate nucleus (b) to muscimol, glycine and β-alanine. The dose-response curves for optic nerve were obtained from separate preparations and the responses were normalized with respect to the response to 1 mM glycine on each preparation. The dose-response curves shown for the cuneate nucleus were obtained by alternating the applications of amino acid to the nucleus end (N) and the caudal end (C) of the dorsal funiculus. The curves for each amino acid were obtained from different preparations and the responses were normalized with respect to the response to 5 μM muscimol on the nucleus end of the dorsal funiculus in each preparation. Each point represents a single response. To the right are tracings of typical responses to glycine and muscimol on optic nerve (c) and the cuneate nucleus (d).

The uptake of glycine into optic nerve was determined by incubating the tissue in Krebs medium containing 4×10^{-7} M [^{14}C]-glycine (specific activity 109 mCi m M^{-1}, (Radiochemical Centre, Amersham) for 30 min at room temperature. The tissue was briefly washed with ice-cold Krebs medium, dissolved in Soluene (Packard) and the radioactivity counted by scintillation spectroscopy.

Results

Glycine and muscimol dose-response relationships

Glycine and muscimol both evoked dose-related depolarizations of the dorsal funiculus fibres in the cuneate nucleus and the fibres of the optic nerve. The responses did not show any fade during the usual 2 min superfusion period, although longer periods of contact with near-maximal concentrations of glycine did result in some fade of the response.

On the optic nerve, the maximal response to glycine was several-fold bigger than the maximal response to muscimol, whereas on the cuneate nucleus, the reverse was the case (Figure 1). On both preparations, the concentrations of glycine required were about one thousand-fold higher than the submaximally effective concentrations of muscimol.

In the foregoing experiments, no distinction was made between the proximal and distal cut ends of the segment of optic nerve but the dorsal funiculus responses all resulted from agonist application to the cuneate nucleus rather than the caudal end of the dorsal funiculus. In further experiments, however, drugs were applied to the caudal end of the dorsal funiculus and it was found that muscimol evoked very similar responses to those in the cuneate nucleus (Figure 1). Glycine evoked distinctly smaller responses at the caudal cut end than at the nucleus end of the dorsal funiculus.

Penetration of glycine into the optic nerve

To obtain some idea of the extent of glycine removal by uptake in the optic nerve and the penetration of glycine along the length of the nerve, various lengths of optic nerve were incubated for 30 min in Krebs medium containing 4×10^{-7} M [^{14}C]-glycine at room temperature. The tissue/medium concentration ratio achieved in 1 mm segments weighing 0.25–0.27 mg was 248 ± 40 (mean ± s.e.mean: $n = 6$). Segments of the size normally used in the electrophysiological experiments (3.5–4.0 mm) accumulated no more [^{14}C]-glycine in total than did the shorter segments so the tissue/medium concentration ratio declined. Thus, it would appear that [^{14}C]-glycine is indeed taken up but only at the cut ends of the optic nerve segment.

Chloride-dependence of the responses to glycine

The effect of replacement of 80% of the chloride in the medium by the less permeant anion isethionate was examined on optic nerves. The low chloride medium was superfused over one end of the optic nerve and the initial effect was a marked shift in the baseline of the record in the hyperpolarizing direction, possibly associated with the establishment of a junction potential. When this had stabilized, responses to glycine in the low chloride medium were found to be reduced to 37% of control while responses to K^+ were more than doubled (Figure 2). Upon restoration of normal Krebs medium, both responses returned to control values.

β-Alanine dose-response relationships

On optic nerve, there was little distinction between the dose-response relationships for β-alanine and glycine. Both were effective over similar concentration ranges although β-alanine evoked a slightly higher maximal response (Figure 1). On the cuneate nucleus, however, the responses to β-alanine were several-fold bigger than those to glycine, over the same effective conentration ranges. Like glycine, β-alanine evoked a smaller response from the caudal end than from the nucleus end of the dorsal funiculus.

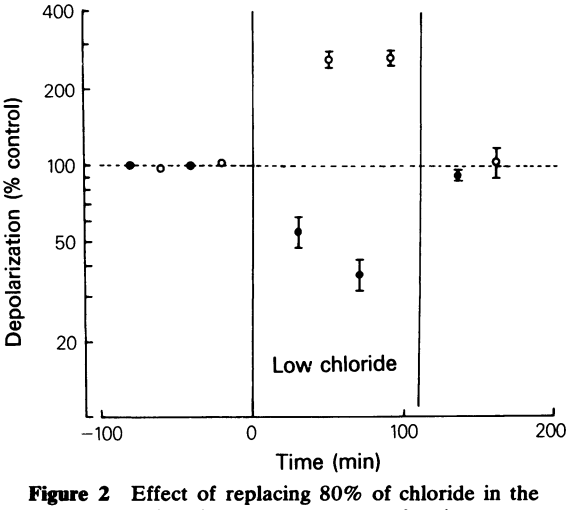

Figure 2 Effect of replacing 80% of chloride in the medium with isethionate on responses of optic nerve to 1 mM glycine (●) and 6 mM K^+ (○). Each point is the mean ± s.e.mean of 4 experiments expressed as percentage of the control mean for each experiment on a logarithmic scale. The standard errors of the control values fall within the diameter of the points.

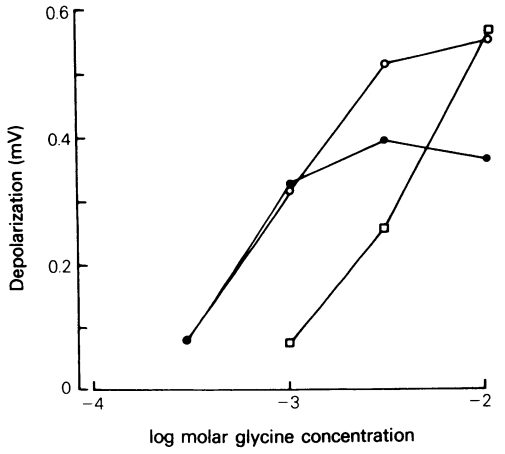

Figure 3 Effect of strychnine on dose-response curves for glycine on the optic nerve. Each point represents a single response to glycine; control (●) and in the presence of strychnine 10^{-7} M (○) and 10^{-6} M (□).

Inactive analogues of glycine

A number of substances were tested for glycine-like activity on the optic nerve in an attempt to find a more potent agonist than glycine or β-alanine. The following substances were tested in concentrations up to 1 mM: α-alanine, aminomethanesulphonic acid, cycloserine, L-proline and nipecotic acid. None of them had detectable glycine-like activity nor did they potentiate or antagonize glycine. β-Proline, 3-pyridine-aminoacetic acid and 3-piperidine-aminoacetic acid were tested in concentrations up to 2 mM and none of them evoked more than a threshold depolarization. 3-Amino-β-proline was rather more active, having about half the potency of glycine, and was antagonized by strychnine to the same extent as was glycine (see below). Taurine evoked modest depolarizations at high concentrations (Simmonds, 1983).

Antagonism of glycine and β-alanine by strychnine

On the optic nerve preparation, 10^{-8} M strychnine was ineffective against glycine; 10^{-7} M strychnine had very little effect on the lower part of the glycine dose-response curve but in 3 of 4 experiments it clearly potentiated the upper part of the curve and increased the maximal response (Figure 3). Strychnine 10^{-6} M caused a parallel displacement to the right of the lower part of the glycine dose-response curve and further increased the maximal response.

It was clear, therefore, that only the lower part of the glycine dose-response curve was suitable for quantitative studies of antagonism. At this level, the Schild plots for strychnine against glycine and β-alanine on optic nerve were very similar (Figure 4). Over the concentration range 10^{-7}–10^{-5} M strychnine both plots were slightly curved but, for the purposes of measurement, the top and bottom halves of the plots were treated as straight lines. The slope of the lower half of the plot with glycine as agonist was not significantly different from 1.00 ($P > 0.1$) but, with β-alanine as agonist, the slope was just significantly less than 1.00 ($P = 0.05$). The slopes of the upper halves of both plots were well below 1.00. pA_2 values for strychnine were 6.58 with glycine as agonist and 6.74 with β-alanine as agonist.

Potency and selectivity of antagonists of glycine, β-alanine and muscimol

Antagonists known to be effective against responses to muscimol in the cuneate nucleus (Simmonds, 1980; 1982) were tested against glycine on the optic nerve. The GABA receptor antagonist bicuculline was without effect at 10^{-5} M but at 10^{-4} M caused a consistently small degree of antagonism of glycine (dose ratio − 1 = 0.57) equivalent to that expected of 1.35×10^{-7} M strychnine. Tubocurarine, which is 10

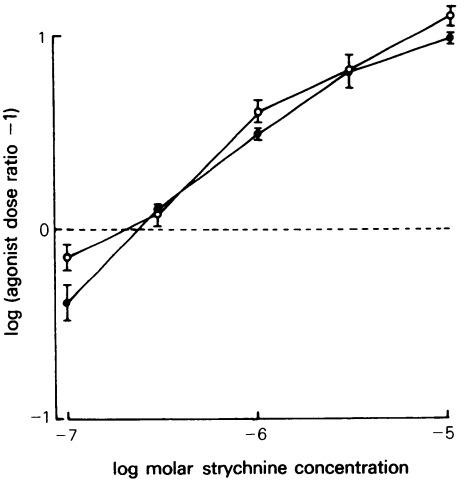

Figure 4 Schild plots for the antagonism of glycine (●) and β-alanine (○) by strychnine on optic nerve. Each point is the mean ± s.e.mean of 4–9 values from separate experiments. Linear regression analysis gave the following slopes: 10^{-7}–10^{-6} M strychnine, 0.882 ± 0.081 ($n = 20$) with glycine as agonist and 0.788 ± 0.097 ($n = 15$) with β-alanine as agonist; 10^{-6}–10^{-5} M strychnine, 0.497 ± 0.053 ($n = 22$) with glycine as agonist and 0.499 ± 0.087 ($n = 16$) with β-alanine as agonist. pA_2 values calculated from 10^{-7}–10^{-6} M strychnine were 6.58 with glycine as agonist and 6.74 with β-alanine as agonist.

Table 1 Profiles of the antagonism of responses to muscimol, glycine and β-alanine on optic nerve and cuneate nucleus (nucleus end of dorsal funiculus)

	Amino acid log dose ratios		
	Muscimol	*Glycine*	*β-alanine*
Optic nerve			
Bicuculline 3×10^{-6} M	0.441* ±0.053 (4)	−0.091* ±0.011 (4)	−0.065 ±0.035 (4)
Picrotoxin 3×10^{-6} M	0.764* ±0.025 (4)	−0.071 ±0.046 (4)	−0.011 ±0.040 (4)
Strychnine 10^{-6} M	−0.016 ±0.009 (4)	0.622* ±0.023 (9)	0.715* ±0.046 (6)
Cuneate nucleus			
Bicuculline 3×10^{-6} M	0.611* ±0.053 (6)	0.005 ±0.019 (4)	†
Picrotoxin 3×10^{-6} M	0.628* ±0.030 (4)	0.016 ±0.015 (4)	†
Strychnine 10^{-6} M	−0.004 ±0.054 (4)	0.441* ±0.027 (12)	0.639*‡ ±0.064 (8)

* Significantly different from zero, $P<0.05$
† Antagonism obtained but displacement of dose-response curve was not parallel
‡ Low response levels only.

times weaker than bicuculline as a GABA receptor antagonist, was ineffective at 10^{-4} M against glycine as were the non-competitive GABA antagonists picrotoxin 10^{-4} M and leptazol 10^{-2} M. All of these GABA antagonists usually caused a small hyperpolarization of the optic nerve.

To compare the pharmacology of the GABA and glycine receptor populations in optic nerve and cuneate nucleus, the profiles of antagonism by bicuculline, picrotoxin and strychnine were determined. Table 1 shows that the general patterns of antagonism of muscimol and glycine were similar on the two tissues, although there were some small quantitative differences. Bicuculline 3×10^{-6} M caused a small but consistent potentiation of glycine on optic nerve.

The antagonism of β-alanine on optic nerve was indistinguishable from that of glycine, strychnine being the only potent antagonist. On the cuneate nucleus, however, β-alanine was antagonized by strychnine, bicuculline and picrotoxin, but in different ways. Strychnine caused a parallel displacement of the lower part of the dose-response curve but higher up the curve the displacement was less (Figure 5). Bicuculline and picrotoxin, on the other hand, failed to displace the lower part of the β-alanine dose-response curve but depressed the upper part of the curve.

Effects of pentobarbitone and flurazepam on responses to glycine

Pentobarbitone 10^{-4} M caused only a slight potentiation of responses to glycine on the optic nerve. The glycine dose-response curve was shifted to the left by 0.068 ± 0.014 log units (mean ± s.e.mean, $P<0.01$). In contrast, the muscimol dose-response curve on optic nerve was shifted to the left by 0.857 ± 0.017

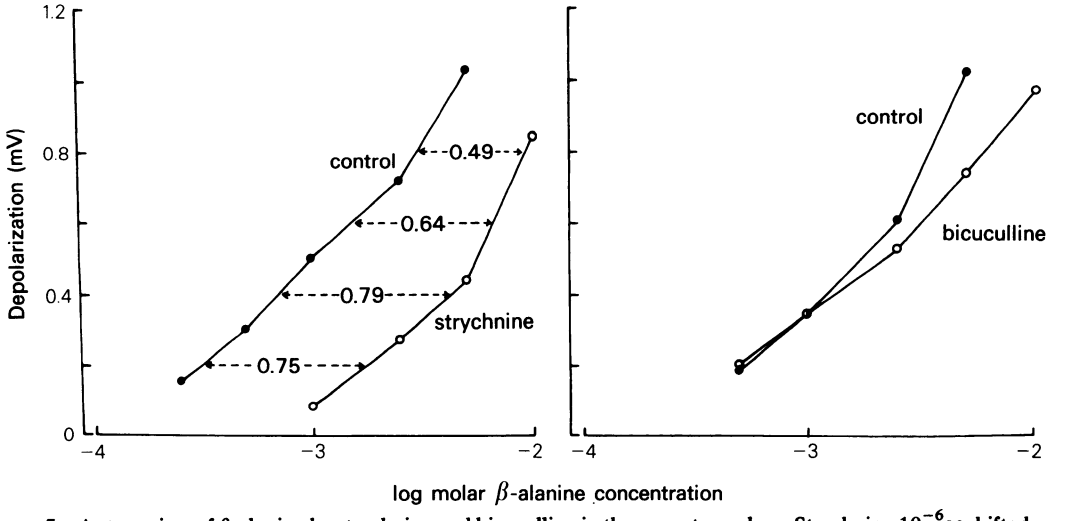

Figure 5 Antagonism of β-alanine by strychnine and bicuculline in the cuneate nucleus. Strychnine 10^{-6} M shifted the lower part of the dose-response curve to the right in a parallel fashion (0.75 − 0.79 log units) but at higher levels of response to β-alanine the antagonism was less. Bicuculline 10^{-6} M did not affect the lower part of the dose-response curve but at higher levels of response to β-alanine a clear antagonism was seen. Each point represents a single response to β-alanine on the nucleus end of the dorsal funiculus.

log units which is very similar to the potentiation of muscimol previously reported in the cuneate nucleus (Simmonds, 1981).

Flurazepam 10^{-6} M had no significant effect on responses to glycine on optic nerve. The muscimol dose-response curve was shifted to the left by 0.290 ± 0.028 log units (mean $\pm$ s.e.mean) which is similar to the potentiation observed in cuneate nucleus.

Discussion

It may seem surprising that segments of myelinated nerves, such as optic nerve and the caudal end of the dorsal funiculus, should show electrophysiological responses to glycine and the GABA analogue muscimol. However, the presence of receptors on axonal membranes has been demonstrated before (Brown & Marsh, 1978; Allan, Evans & Johnston, 1980) and drugs will gain access to the receptors where the myelination has been disrupted at the cut ends. The consequent depolarizations to glycine and muscimol imply that the receptors exist in functional complexes with their ionophores at the re-sealed ends of the axons. All of these receptor complexes are extrasynaptic, in contrast with those on the nerve terminals of the dorsal funiculus which are probably a mixture of synaptic and extrasynaptic, at least for GABA (Levy, 1977). Chloride ionophores are involved in both glycine and GABA receptor responses, as indicated by the similar chloride-dependence of glycine responses on optic nerve and GABA responses on the dorsal funiculus (Simmonds, 1978). The fact that the responses to both glycine and GABA receptor activation are depolarizing rather than hyperpolarizing suggests that the electrochemical gradient for chloride is outward in those isolated neuronal preparations.

The pharmacological properties of the GABA receptor and ionophore complex activated by muscimol have already been described in some detail in the cuneate nucleus slice (Pickles & Simmonds, 1980; Simmonds, 1980; 1981; 1982). Key features tested on the optic nerve showed a close similarity of the GABA receptor complexes on these two preparations. Thus, the potencies of (+)-bicuculline and picrotoxin as competitive and non-competitive antagonists, respectively, and pentobarbitone and flurazepam as potentiators of muscimol, together formed matching profiles on optic nerve and cuneate nucleus.

A similar comparison of the pharmacological profiles of glycine responses was made. Of the drugs tested, only the antagonist strychnine had more than a marginal effect and its potency on the optic nerve was slightly greater than in the cuneate nucleus. It is possible that this small difference was due to a strychnine-resistant component of the action of glycine (Pickles, 1979) which might be more apparent in the small responses of the cuneate nucleus to glycine. Although the positive evidence is limited, there is nothing to suggest that the glycine receptor complexes are different in these two preparations.

There was a marked difference between optic nerve fibres and the dorsal funiculus fibres in the cuneate nucleus with regard to the maximal responses to glycine and muscimol. The simplest explanation for this is that there exists a higher density of glycine receptors than GABA receptors on optic nerve fibres and *vice versa* on dorsal funiculus fibres. In cultured spinal neurones from the mouse, the conductance of the chloride ionophores operated by glycine is twice that of the chloride ionophores operated by GABA and muscimol (Barker, MacDonald, Mathews, McBurney & Oertel, 1981). A similar situation in optic nerve would account for only about 20% of the difference between maximal responses to glycine and muscimol whilst in the cuneate nucleus it would lead to an under estimate of the difference in receptor densities.

In addition, there was a small difference between the rostral end of the dorsal funiculus within the cuneate nucleus and the caudal end of these fibres. The responses to glycine were about twice as big on the rostral end as on the caudal end. This could be due to higher numbers of glycine receptors on the terminals than on the cut ends of the axons in the dorsal funiculus. Alternatively, the glycine receptors may reside predominantly on the few cuneate nucleus neurones which project caudally in the dorsal funiculus (Burton & Loewy, 1977) with a greater number of receptors on the cell bodies than on the cut ends of the axons of those neurones.

Just as GABA is not the ideal agonist to use in quantitiative pharmacological studies of the GABA receptor (Simmonds, 1980), so glycine is not ideal for such studies of the glycine receptor. The uptake processes for glycine will diminish its free concentration in the tissue and this will affect the apparent potencies of antagonists and distort their Schild plots (Kenakin & Beek, 1981). Also, the high concentrations of glycine required under control conditions allow only modest degrees of antagonism to be measured if the glycine concentrations are to remain within reasonable limits. What is required is an analogue that is more potent than glycine and that is not a substrate for uptake processes. None of the analogues tested met these requirements so it remained for the detailed evaluation of strychnine on optic nerve to be made against glycine.

The lowest effective concentrations of strychnine did not antagonize glycine but rather potentiated responses at the top of the dose-response curve. This

may represent a block of desensitization to glycine and is reminiscent of the effects of low concentrations of both bicuculline and strychnine on GABA dose-response curves in the cuneate nucleus (Simmonds, 1978). With increasing concentrations of strychnine, the parallel shift to the right of the glycine dose-response curve generated a non-linear Schild plot. Although this does not conform with the required straight line of slope = 1.00 for competitive antagonism, the deviation does not necessarily mean that strychnine is not a competitive antagonist of glycine, for the reasons given above. A similar problem was encountered with bicuculline antagonism of GABA (Simmonds, 1980) and resolved with the use of muscimol. Nevertheless, at the low level of antagonism that determines pA_2 the values for bicuculline against GABA and muscimol were similar. It is possible, therefore, that the pA_2 value of 6.58 for strychnine against glycine is close to the real pA_2 value for strychnine against glycine receptor-mediated responses. This yields a selectivity ratio of 19.5 for strychnine as a glycine antagonist compared with GABA receptor antagonism (Simmonds, 1982). In contrast, bicuculline has a selectivity ratio of 156 as a GABA receptor antagonist compared with antagonism of glycine.

The responses to β-alanine on optic nerve and their antagonism by strychnine indicated that β-alanine and glycine operated on similar, if not the same, receptors. The two amino acids were of similar potency and their antagonism by strychnine yielded similar pA_2 values. In the cuneate nucleus, however, an extra effect of β-alanine was seen. The strychnine-sensitive component was present over the entire range of the dose-response curve and in addition, a bicuculline- and picrotoxin-sensitive component was apparent at the higher concentrations of β-alanine. The concentrations of β-alanine required for this latter component were similar to those that activate the GABA receptors on the rat superior cervical ganglion (Bowery & Brown, 1974). Thus, in accord with earlier observations *in vivo* (Curtis *et al.*, 1971), it appears that β-alanine may activate both glycine and GABA receptors, slightly lower concentrations being required to activate glycine receptors.

The close similarity in properties between the glycine receptors on optic nerve and those studied *in vivo* (Curtis *et al.*, 1971; Hill *et al.*, 1976) suggests that the rat optic nerve preparation will be a useful *in vitro* system for quantitative studies of the mammalian neuronal glycine receptor plus ionophore complex.

I am grateful to Diana Barlow for her skilled technical contributions to this work. Samples of β-proline, 3-amino-β-proline, 3-piperidine-aminoacetic acid and 3-pyridine-aminoacetic acid were kindly donated by Dr P. Jacobsen of the Royal Danish School of Pharmacy, Copenhagen.

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