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Antagonism of inhibitory amino acids by the steroid derivative RU5135

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1 The steroid derivative RU5135 has been tested for its ability to antagonize glycine and the γ-aminobutyric acid (GABA) analogue muscimol on isolated preparations of rat optic nerve and cuneate nucleus, respectively.

2 On the cuneate nucleus, RU5135 antagonized muscimol in a competitive manner with a pA_2 value of 8.31. RU5135 shared a common site of action with bicuculline that was separate from the picrotoxin site.

3 On the optic nerve, RU5135 antagonized glycine with a pA_2 of 7.67. It shared a common site of action with strychnine.

Introduction

Receptor binding studies have shown that the steroid derivative RU5135 (3α-hydroxy-16-imino- 5β-17-aza-androstan-11-one) has a high affinity for both γ-aminobutyric acid (GABA) and glycine receptors. Since RU5135 inhibits GABA-stimulated [^{3}H]-diazepam binding, it appears that RU5135 is a GABA receptor antagonist, about 500 times more potent than bicuculline (Hunt & Clements-Jewery, 1981). These authors also cite a brief abstract reporting that RU5135 causes convulsions but is devoid of any hormonal activity in rodents. In another study, RU5135 has been shown to induce epileptiform activity (Myslobodsky & Kofman, 1983).

The purpose of the present experiments was to evaluate RU5135 as an antagonist of electrophysiological responses to the GABA analogue muscimol and to glycine.

Methods

The experiments were performed on slices of rat medulla oblongata, containing the dorsal funiculus and cuneate nucleus (Simmonds, 1978; 1980) and the rat optic nerve (Simmonds, 1983). The cuneate nucleus slices and optic nerves were placed in similar two-compartment baths so that the dorsal funiculi and the optic nerves, respectively, projected through a greased slot in the barrier between the compartments. The preparations were superfused with Krebs medium at 20°C. Drug solutions diluted in the Krebs medium were perfused through one compartment only, that containing the terminals of the dorsal funiculus in the case of the cuneate nucleus or either end of the optic nerve. 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 nerve fibres that passed through the barrier. The responses were measured at their peak amplitudes.

Muscimol (Fluka) was used routinely as the agonist for GABA receptors on the cuneate nucleus slice and glycine (Koch Light) as the glycine receptor agonist on the optic nerve. In order to minimize the problem of desensitization, agonist doses on the lower half of the dose-response curve were selected. The doses used under control conditions were usually 2.5 μM and 5 μM for muscimol and 0.5 mM and 1 mM for glycine. The agonists were superfused for periods of 2 min. The prospective antagonists were superfused for 30–40 min before and then during the redetermination of agonist responses bridging the same response range as the control line. From the parallel shifts of the dose-response curves, equipotent dose-ratios for muscimol and glycine were calculated in the presence of each concentration of antagonist.

The data for RU5135 were plotted as Schild plots of log (agonist dose-ratio − 1) against log (RU5135 concentration). In order to determine whether RU5135 was acting at the same site as bicuculline or

picrotoxin to antagonize muscimol and at the same site as strychnine to antagonize glycine, RU5135 was tested in combination with each of these antagonists (Simmonds, 1980). With each pair of slices or nerves from the same animal, RU5135 was tested first on one slice, the other antagonist first on the other slice and then the combination of the two antagonists on both slices. The concentrations of the antagonists were selected to give individual dose-ratios of about 5. This should allow a clear separation between the predicted dose-ratio (DR) for the combination of antagonists which would be $DR_1 + DR_2 - 1$ (assuming slopes of 1 for the individual Schild plots) for a common site of action and $DR_1 \times DR_2$ for independent sites of action (Barlow, 1980). For each pair of tissues the predicted dose-ratios for the combination of antagonists were calculated from the values obtained for each antagonist alone and reference to the Schild plot for RU5135. These data from replicate experiments were grouped and tested for significance by Mann-Whitney U-test.

Bicuculline (Sigma) was prepared as a 10 μM solution in 0.02 M HCl and RU5135 (Roussel Uclaf) as 1 mM and 10 μM solutions in ethanol. They were diluted into the Krebs medium just before use. The final concentration of ethanol never exceeded 0.1% and, at this concentration, ethanol has no effect on amino acid responses (unpublished data). Picrotoxin (Sigma) and strychnine (Hopkin & Williams) were dissolved directly in the Krebs medium. The Krebs medium contained (mM): NaCl 118, KCl 2.1, KH_2PO_4

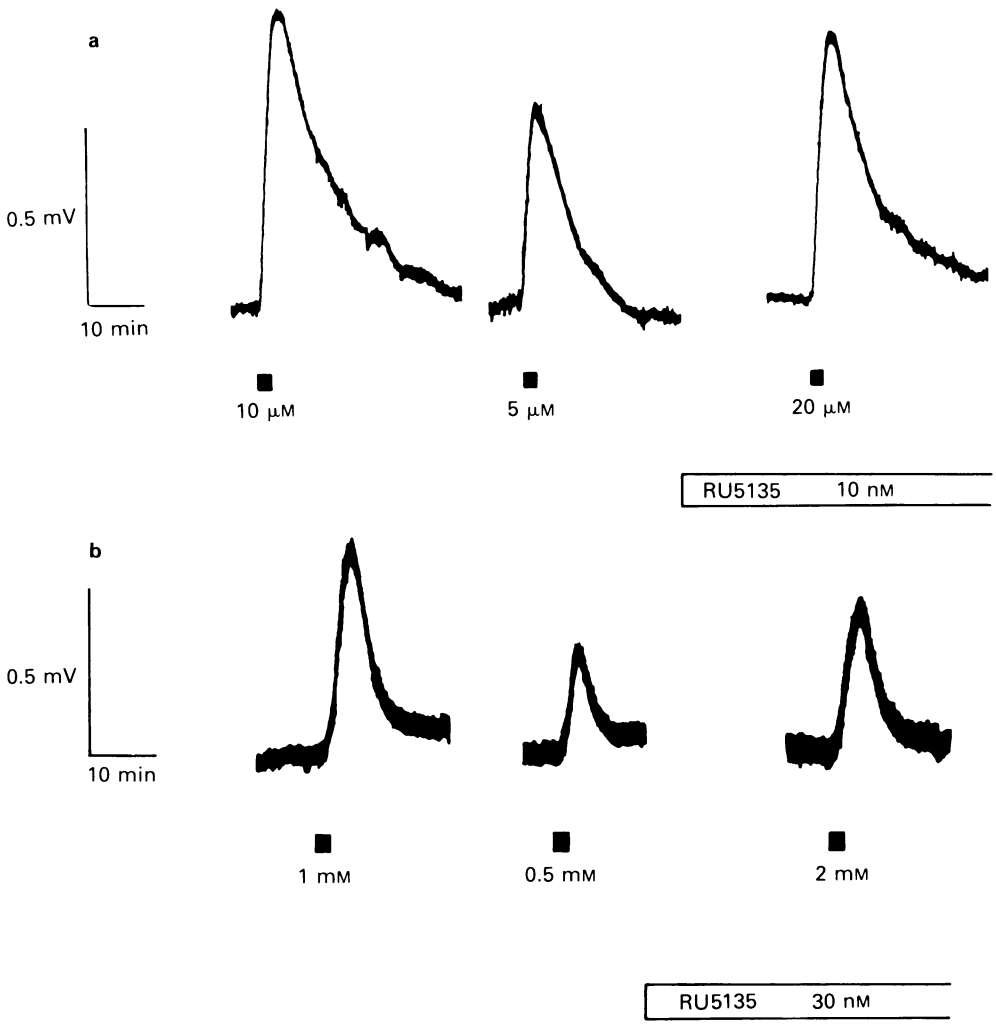

Figure 1 Responses of the cuneate nucleus (a) and optic nerve (b) to single doses of muscimol and glycine, respectively, and the effect of RU5135.

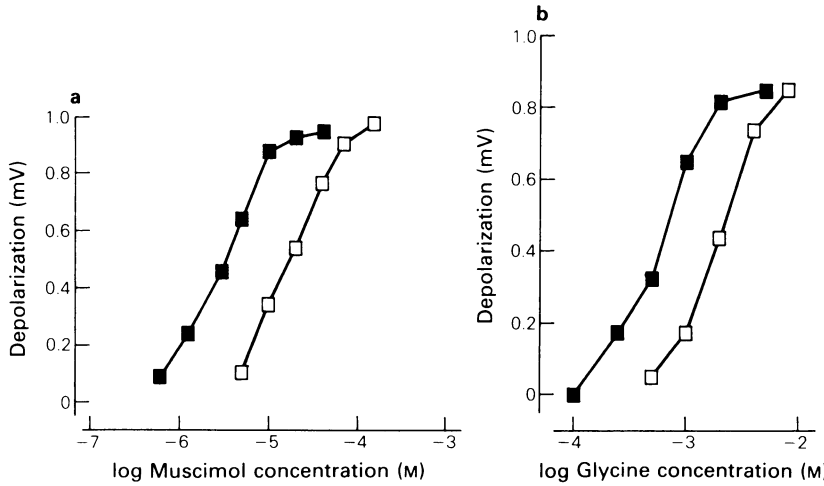

Figure 2 Dose-response curves showing the antagonism, by RU5135 (30nM), of (a) muscimol and (b) glycine on the cuneate nucleus and optic nerve, respectively, in single experiments (control ■, RU5135 □). RU5135 shifted both dose-response curves to the right in a parallel fashion without depressing the maxima.

1.2, $CaCl_2$ 2.5, $MgSO_4$ 2.2, $NaHCO_3$ 25, and glucose 11, and was continuously gassed with 95% O_2 and 5% CO_2.

Results

Responses to muscimol and glycine and the direct effects of RU5135

Superfusion of the cuneate nucleus and optic nerve with muscimol and glycine, respectively, produced a depolarization of nerve fibres in these preparations (Figure 1). This depolarization was dose-dependent in nature (Figure 2). The introduction of RU5135 into the Krebs medium superfusing the drug side of the bath had no consistent effect on the baseline recording.

Antagonism of muscimol by RU5135

In all experiments, RU5135 displaced the lower part of the muscimol dose-response curve in an approximately parallel fashion. When full dose-response curves were constructed, RU5135 did not depress the maximum response (Figure 2a).

A Schild plot of log (muscimol dose-ratio -1) versus log (RU5135 concentration) was constructed from the lower part of the dose-response curves (Figure 3). The data could be represented as a straight line relationship for which the calculated slope (linear regression analysis) was 1.12 ± 0.062 ($n = 40$). This slope was not significantly different from one (t test, $P > 0.05$). The calculated pA_2 was 8.31 ± 0.033. For comparison, the previously obtained Schild plot for bicuculline (Simmonds, 1980) is included in Figure 3 along with data for 6×10^{-6}M bicuculline obtained during the present experiments. These results suggest that a valid comparison can be made between the previous Schild plot for bicuculline (slope = 1.07, $pA_2 = 5.98$) and that obtained in the present experiments for RU5135. Thus, RU5135 was 214 times more potent than bicuculline as an antagonist of muscimol.

Antagonism of glycine by RU5135

RU5135 displaced the lower part of the glycine dose-response curve in an approximately parallel fashion

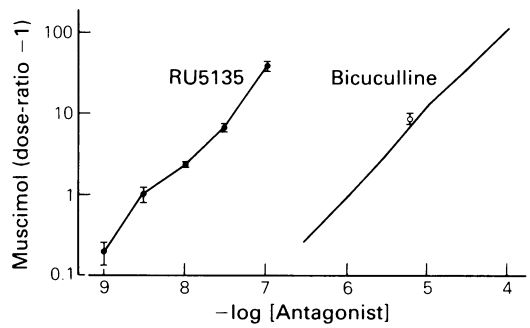

Figure 3 Schild plots of log (muscimol dose-ratio − 1) versus log (antagonist concentration) obtained on slices of rat cuneate nucleus. For RU5135, each point is the mean of 5–12 values. For bicuculline, the line represents the Schild plot taken from Simmonds (1980) and the open circle is the mean of 5 values obtained in the present series of experiments; vertical lines indicate s.e.mean.

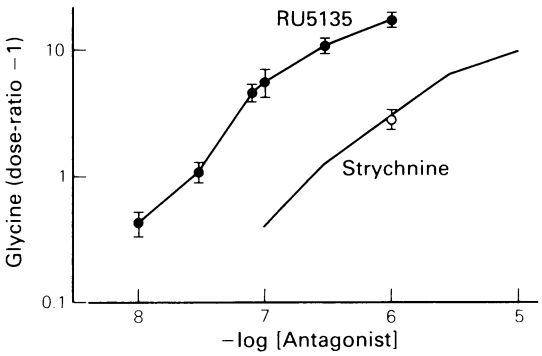

Figure 4 Schild plots of log (glycine dose-ratio − 1) versus log (antagonist concentration) obtained on rat optic nerve. For RU5135, each point is the mean of 5–7 values. For strychnine, the line represents the Schild plot taken from Simmonds (1983) and the open circle is the mean of 4 values obtained in the present series of experiments; vertical lines show s.e.mean.

and, as with muscimol, did not depress the maximum of the full dose-response curve (Figure 2b).

A Schild plot of log (glycine dose-ratio − 1) versus log (RU5135 concentration) was constructed from the lower part of the dose-response curves (Figure 4). Overall the plot had a slope of 0.84 ± 0.066 ($n = 34$) which was significantly less than 1 and appeared to be slightly curved. For the purposes of calculating a pA_2 value, the lower half between 10^{-8} and 10^{-7}M RU5135 was treated as a straight line. This gave a slope of 0.86 ± 0.184 ($n = 21$) which was not significantly different from one ($P > 0.05$) and pA_2 value of 7.67 ± 0.068. For comparison, the previously obtained Schild plot for strychnine (slope = 0.88) (Simmonds, 1983) is included in Figure 4 together with data for 10^{-6}M strychnine obtained during the present experiments. These results indicate that the potency of strychnine remained the same as before and that the shapes of Schild plots for RU5135 and strychnine were similar. Comparison of the pA_2 value for strychnine (6.58) with that for RU5135 indicates that RU5135 is 15 times more potent than strychnine as an antagonist of glycine.

Combinations of RU5135 with other antagonists

These experiments were performed to determine

Table 1 Antagonism of muscimol and glycine by antagonists in combination

	Observed dose-ratios Antagonists		*Predicted dose-ratios for combinations*	
	Alone	*Combination*	*Common site*	*Separate site*
(a) *Cuneate slice*				
RU5135	8.5			
(3×10^{-8}M)	4.7–12.7			
	(5)	22.5	18.9	84.6
		12.7–30.4	12.5–26.6	38.5–163.1
Bicuculline	10.1	(10)	$P > 0.05$	$P < 0.01$
(6×10^{-6}M)	7.3–14.5			
	(5)			
RU5135	3.3			
(1×10^{-8}M)	2.6–3.9			
	(5)	29.6	11.5	28.2
		21.2–48.4	10.4–14.1	21.6–37.1
Picrotoxin	8.5	(10)	$P < 0.01$	$P > 0.1$
(1×10^{-5}M)	7.0–11.0			
	(5)			
(b) *Optic nerve*				
RU5135	5.5			
(8×10^{-8}M)	3.8–8.0			
	(4)	9.0	8.6	27.6
		4.4–13.5	6.8–11.5	14.4–44.0
Strychnine	4.9	(8)	$P > 0.1$	$P < 0.01$
(1×10^{-6}M)	3.8–5.8			
	(4)			

The values are the means and ranges of the agonist dose-ratios for the numbers of experiments shown in parentheses. The observed and predicted dose-ratios, were compared using Mann-Whitney U-test and the statistical significances of the differences are given under the predictions.

whether RU5135 acts at a common site with bicuculline to antagonize muscimol and at a common site with strychnine to antagonize glycine. The results are shown in Table 1.

For the antagonism of muscimol, the dose-ratios obtained with RU5135 and bicuculline were significantly different ($P < 0.01$) from the values predicted for separate sites of action and very close to the values predicted for a common site of antagonist action. Antagonism of muscimol by RU5135 and picrotoxin gave dose-ratios that were significantly different ($P < 0.01$) from the values predicted for a common site of antagonist action and close to the values predicted for separate sites of action.

For the antagonism of glycine, the dose-ratios obtained with RU5135 and strychnine were significantly different ($P < 0.01$) from the values predicted for separate sites of action and very close to the values predicted for a common site of antagonist action.

Discussion

The results indicate that RU5135 behaves like bicuculline (Simmonds, 1980; 1982) as an antagonist of muscimol but it is 214 times more potent than bicuculline. The arguments for bicuculline being a competitive antagonist at the $GABA_A$ receptor have been made before (Simmonds, 1982) and the same conclusion can be drawn for RU5135. Thus, the slope of the Schild plot close to 1, and a common site of action with bicuculline, but separate from picrotoxin, represent the best evidence that can be obtained from dose-response studies for RU5135 being a competitive antagonist at the $GABA_A$ receptor. This conclusion is further supported by the displacement of [^{3}H]-GABA binding by RU5135 (Hunt & Clements-Jewry, 1981).

At the glycine receptor, RU5135 behaved like strychnine (Simmonds, 1983) but it was 15 times more potent than strychnine. The Schild plots for both antagonists were similarly shaped and the antagonists clearly acted at a common site. Whilst these data do not conform strictly with the theoretical predictions for competitive antagonism since the Schild plot is non-linear, the deviation may well be due to the operation of uptake processes for glycine (Simmonds, 1983). However, this cannot be demonstrated at present owing to the lack of a specific inhibitor of glycine uptake.

The pA_2 values for RU5135 against muscimol and glycine indicate a rather small degree of selectivity, RU5135 being 3.6 times more potent at the $GABA_A$ receptor than at the glycine receptor. This contrasts with a selectivity ratio of 156 for bicuculline at these receptors and a selectivity ratio of 19.5 for strychnine as a glycine antagonist compared with its antagonism at the $GABA_A$ receptor (Simmonds, 1983). Nevertheless, RU5135 is the most potent antagonist of both $GABA_A$ receptors and glycine receptors that we have tested.

The relevance of these observations to the convulsant action of RU5135 is not clear. The concentrations of RU5135 required to antagonize $GABA_A$ and glycine receptors are well below the likely circulating concentrations of RU5135 following intraperitoneal injection of $2\,mg\,kg^{-1}$ to induce seizure activity (Myslobodsky & Kofman, 1983). However, the concentration of RU5135 achieved in the brain following intraperitoneal injection has not been measured.

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