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Inhibition of γ-aminobutyric acid uptake by bicuculline analogues

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Abstract

Enantiomers of *nor*bicuculline, (+)[1*S*,9*R*] and (−)[1*R*,9*S*]*erythro*-1-[1′-(4′,5′-methylenedioxyphthalidyl)]-6,7-methylenedioxy-1,2,3,4-tetrahydroisoquinoline and of the *N*-methyl derivatives {(+)[1*S*,9*R*] and (−)[1*R*,9*S*]bicuculline} were found to inhibit the progress of the γ-aminobutyric acid transporter-mediated uptake of 40 μM [^{14}C]γ-aminobutyric acid into native plasma membrane vesicles from the rat cerebral cortex at 30°C. The values for the dissociation constants of the reversible inhibition, relative to (+)[1*S*,9*R*]bicuculline, in order of increasing inhibition, were: (−)[1*R*,9*S*]bicuculline, 3.3; (+)[1*S*,9*R*]-bicuculline, 1.0; (−)[1*R*,9*S*]*nor*bicuculline, 0.4 ≈ (+)[1*S*,9*R*]*nor*bicuculline; guvacine, 0.02. The *nor*bicucullines have higher potencies than (+)[1*S*,9*R*]bicuculline for the γ-aminobutyric acid transporter, in contrast to the relative potencies of these inhibitors for the inhibition of function and γ-aminobutyric acid binding of the γ-aminobutyric acid type A receptor. © 1997 Elsevier Science B.V.

Keywords: Bicuculline analogue, enantiomer; [^{14}C]GABA ([^{14}C]γ-aminobutyric acid); Uptake, quench flow; Cortex, rat

1. Introduction

Biological evaluation of the effect of N-methyl substitution of bicuculline-related antagonists on their effect on the binding and function of the $GABA_A$ receptor was made possible by the stereoselective synthesis of (+)[1*S*,9*R*] and (−)[1*R*,9*S*]*erythro*-1-[1′-(4′,5′-methylene-dioxyphthalidyl)]-6,7-methylenedioxy-1,2,3,4-tetrahydroisoquinoline {(+)[1*S*,9*R*] and (−)[1*R*,9*S*]*nor*bicuculline} (Kardos et al., 1996). We now report on the effect of these compounds on GABA uptake. The abstract of preliminary results has been published elsewhere (Kardos et al., 1995).

2. Materials and methods

The stereoselective synthesis of norbicuculline enantiomers has been described recently (Kardos et al., 1996). Guvacine was from Research Biochemicals International (Natick, MA, USA). [^{14}C]GABA with specific activity, 8.04 mCi/mmol was from the Isotope Institute (Budapest, Hungary). All other drugs and reagents were from Sigma (St.Louis, MO, USA). Stock solutions of bicuculline derivatives in dimethyl sulfoxide (DMSO) were prepared freshly and stored in the dark on ice until use. Male rats (160–200 g body weight) were from LATI (Gödöllő, Hungary).

The rats were guillotined and the brain was removed rapidly, rinsed and placed in ice-cold physiological saline and the cortex was dissected out. Native plasma membrane vesicle suspensions were prepared from approximately 0.6 g cortical tissue in 10 mM HEPES buffered physiological salt solution (in mM: NaCl, 145; KCl, 5; $MgCl_2$, 1; $NaHCO_3$, 2; ATP, 1; D-glucose, 10; aminooxyacetate, 0.1; pH 7.5) after the method of Serfőző and Cash (1992). Protein concentration measured by the Folin-phenol reagent method (Lowry et al., 1951) was adjusted to 0.90 mg protein/ml. The quench flow technique (Cash and Subbarao, 1987a,b; Cash et al., 1991) was used to measure GABA uptake as described previously (Kardos et al., 1994). The membrane vesicle suspension was preincubated with the inhibitors (or only 0.5% DMSO) for 10 min at 30°C followed by cooling to 0°C (3 min). Briefly, 300 μl

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of each reactant was loaded into the machine and pre-warmed (3 min at 30°C). After fast (< 2 ms) mixing of equal volumes (300 μl) of [^{14}C]GABA solution and the membrane suspension, the mixture was incubated for different periods of time at 30°C. The reaction was quenched rapidly (< 3 ms) by 300 μl uptake inhibitor, guvacine (2 mM after mixing), the mixture was passed through a glass fibre filter disk (Whatman GF/B) and the membranes were retained on the filter washed with 2 × 5 ml of ice-cold buffer in 15 s.

The progress of transmembrane flux of 40 μM [^{14}C]GABA was followed over a 1–700 s time range in the absence or presence of various concentrations of guvacine, *nor*bicuculline and bicuculline enantiomers (0–150 μM inhibitors, I). Duplicate measurements were made at each time point. In the absence or presence of the inhibitors, the uptake followed a single first-order approach to a final value (M_∞) with a rate constant (k). The final uptake value was independent of [I]. The relative dissociation equilibrium constants for inhibition were determined by fitting the GABA uptake rate constants, measured with different inhibitor concentrations, to equations for non-competitive, $k = k_{max}[U][G]/\{(K_d + [G])(1 + [I]/K_i)\}$ and competitive, $k = k_{max}[U][G]/\{K_d(1 + [I]/K_i) + [G]\}$ inhibition (the relative values of K_i do not depend on the mechanism). k_{max} is the saturation rate constant; [G] the concentration of [^{14}C]GABA ([G] = 40 μM) and U represents the uptake sites per unit internal volume ([U] = 1 taken as customary). K_d is the dissociation equilibrium constant for GABA if the first GABA uptake step after binding is the slowest step. This was taken to be $K_d = 4.3$ μM (Kardos et al., 1994) for this calculation (Fig. 4).

3. Results

The progress of the specific uptake of 40 μM [^{14}C]GABA into native plasma membrane vesicle suspensions from the rat cerebral cortex at 30°C, pH 7.5 was followed up to the final value which was reached in about 500 s with no inhibitor present (Fig. 1). Fitting a single, first-order phase of uptake, the average rate constants, k and final influx values, M_∞ from repeated individual measurements were $k = 4.45\ (\pm 0.84) \times 10^{-3}\ s^{-1}$ and $M_\infty = 3.52 \pm 0.09$ nmol [^{14}C]GABA/mg of protein, respectively. With pooled data (6 experiments) similar values, $k = 4.44\ (\pm 0.24) \times 10^{-3}\ s^{-1}$ and $M_\infty = 3.54 \pm 0.09$ nmol [^{14}C]GABA/mg of protein were obtained, suggesting that the rate and extent of uptake were not dependent on the batch of native plasma membrane vesicles. Assuming the reported value of the internal volume to which GABA has access (3.8 ± 0.7 μl/mg of protein, Kanner, 1978) the calculated internal GABA concentration would have been approximately 1 mM. This represents a concentration gradient of at least 20-fold. In the presence of

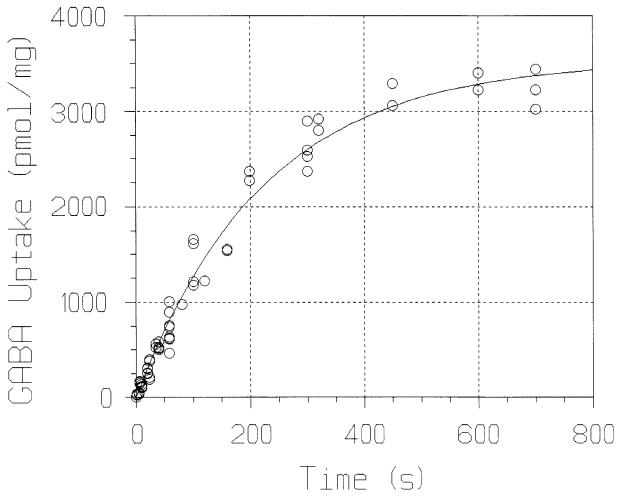

Fig. 1. Progress of the specific uptake of 40 μM [^{14}C]GABA into native plasma membrane vesicle suspensions at 30°C, pH 7.5, in the presence of 0.5% DMSO. Pooled data from measurements with 6 different preparations. The line was computed assuming first-order kinetics with a single uptake process with rate constant, $k = 4.44\ (\pm 0.24) \times 10^{-3}\ s^{-1}$ and final influx value $M_\infty = 3.54 \pm 0.09$ nmol [^{14}C]GABA/mg of protein.

(−)[1*R*,9*S*]*nor*bicuculline the final influx value was the same as that with no inhibitor (Fig. 2A).

Inhibition of GABA uptake by the various bicuculline derivatives is illustrated in Figs. 2 and 3. When present in

Table 1
Inhibition of specific [^{14}C]GABA uptake into native plasma membrane vesicles from the rat cerebral cortex at 30°C, pH 7.5

Inhibitor	Concentration (μM)	Uptake rate constant $k \times 10^{-3}$ (s^{-1})
No inhibitor	—	4.44 ± 0.24
Guvacine	5	1.64 ± 0.20
	20	0.27 ± 0.06
(−)[1*R*,9*S*]*Nor*bicuculline	20	2.71 ± 0.12
	50	2.04 ± 0.09
	150	0.80 ± 0.20
(+)[1*S*,9*R*]*Nor*bicuculline	50	2.98 ± 0.30
	150	0.36 ± 0.13
(+)[1*S*,9*R*]Bicuculline	50	4.13 ± 0.31
	150	2.00 ± 0.27
	150	1.60 ± 0.21
(−)[1*R*,9*S*]Bicuculline	150	3.29 ± 0.15
	150	3.42 ± 0.16

Membrane vesicle suspensions were preincubated with or without the drug tested at different concentrations. Aliquots of the preincubated suspensions (300 μl) were rapidly mixed with an equal volume of [^{14}C]GABA solution (40 μM final, 8.04 mCi/mmol), with or without the drug in Hepes buffered physiological salt solution. At the end of incubation the mixture was rapidly mixed with a quench-solution containing 6 mM guvacine in the above buffer (2 mM after mixing) and filtered. The progress of uptake was followed between 1–700 s with duplicate determinations at stated time points (5–7). Non-specific uptake was determined in the presence of 150 μM guvacine. Rate constants (k = mean ± S.D.) were determined by fitting untransformed data (10–14) with least squares analysis. Rate constants from different experiments were compared on the basis of pooled data (64) giving, with no inhibitor, $k = 4.44\ (\pm 0.24) \times 10^{-3}\ s^{-1}$ and $M_\infty = 3.54 \pm 0.09$ nmol [^{14}C]GABA/mg of protein.

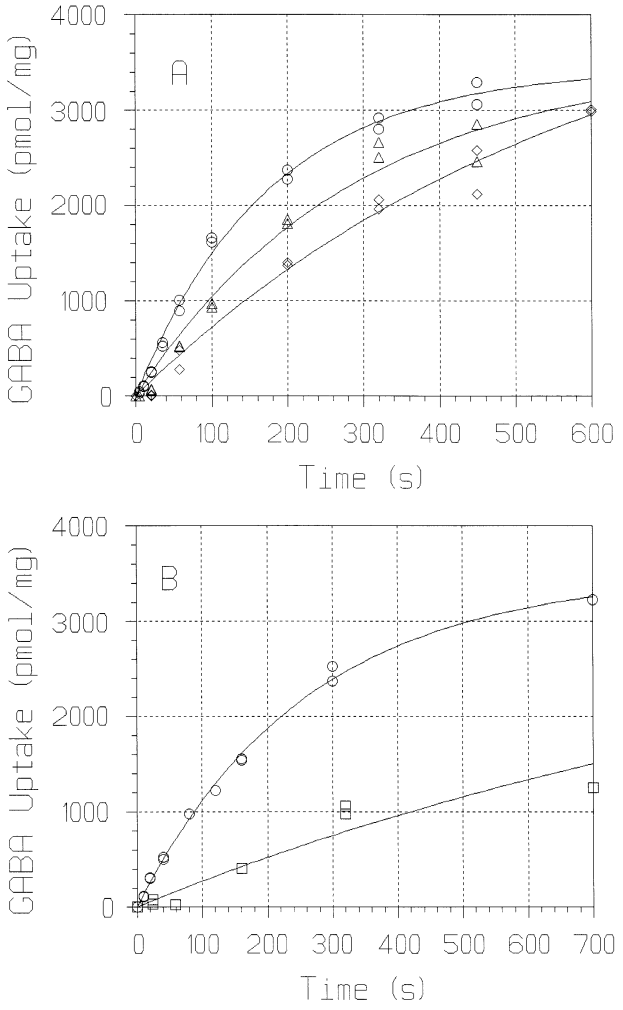

Fig. 2. Effect of (−)[1*R*,9*S*]*nor*bicuculline on the specific uptake of 40 μM [^{14}C]GABA into native plasma membrane vesicle suspension at 30°C, pH 7.5, 0.5% DMSO. Lines were computed applying first-order kinetics with one uptake process. (A) No inhibitor (○); 20 μM (−)[1*R*,9*S*]*nor*bicuculline (△); 50 μM (−)[1*R*,9*S*]*nor*-bicuculline (◇); (B) No inhibitor (○); 150 μM (−)[1*R*,9*S*]*nor*bicuculline (□).

Table 2
Relative inhibition equilibrium constants of bicuculline analogues and guvacine for GABA transport

Inhibitor	Relative K_i [a]
Guvacine	0.02
(−)[1*R*,9*S*]*Nor*bicuculline	0.4
(+)[1*S*,9*R*]*Nor*bicuculline	≈ 0.4
(+)[1*S*,9*R*]Bicuculline	1.0
(−)[1*R*,9*S*]Bicuculline	3.3

[a] Relative K_i was calculated (see Section 2) from the dependence of GABA uptake rate on inhibitor concentration. Curve-fitting of data (Figs. 2–4) was performed with the least squares method using SCIENTIST ver 2.03. The rate parameters and the equilibrium dissociation constants of inhibition are given as means ± S.D. and were analysed using one-way analysis of variances (ANOVAs, ORIGIN ver 3.5) for the post hoc comparisons. A value of $P < 0.05$ was considered significant. K_i for (+)[1*S*,9*R*]bicuculline was taken to be 1.0.

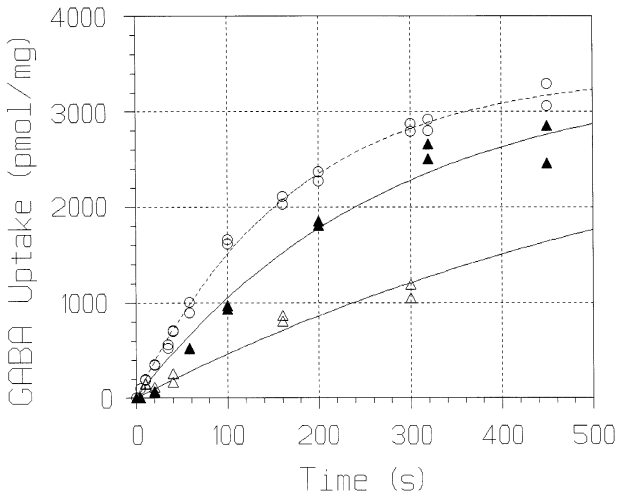

Fig. 3. Comparison of the inhibitory effects of (−)[1*R*,9*S*]*nor*bicuculline and guvacine on the specific uptake of 40 μM [^{14}C]GABA into native plasma membrane vesicle suspensions at 30°C, pH 7.5, 0.5% DMSO. Lines were computed applying first-order kinetics with one uptake process. No inhibitor (○); 20 μM (−)[1*R*,9*S*]*nor*bicuculline (▲); 5 μM guvacine (△).

a concentration of 20 μM, (−)[1*R*,9*S*]*nor*bicuculline inhibited [^{14}C]GABA uptake much less effectively than did 5 μM guvacine (Fig. 3). Increased inhibition (decrease of k) was observed (Table 1) with increasing concentrations of the enantiomers of *nor*bicuculline and bicuculline (20–150 μM) as well as by guvacine (5–150 μM). The relative values of the reversible inhibition constants given in Table 2 were determined from the dependence of the inhibition rates on inhibitor concentration (Fig. 4).

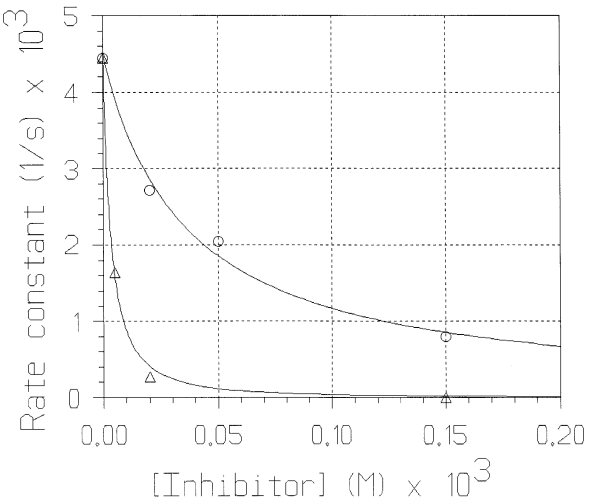

Fig. 4. Dependence of GABA uptake rate on inhibitor concentration. Rate constants of the specific uptake of 40 μM [^{14}C]GABA into native plasma membrane vesicle suspension at 30°C, pH 7.5, 0.5% DMSO were fitted by a scheme of reversible inhibition (Section 2), to give relative values for the inhibition constants, K_i (Table 2). [1*R*,9*S*]*Nor*bicuculline, (○); guvacine, (△).

4. Discussion

Bicuculline analogues were found to inhibit GABA uptake, and the relative values of the inhibition equilibrium constants of the *nor* bicuculline enantiomers and bicuculline enantiomers were determined.

When comparing bicuculline analogues and guvacine we found that bicuculline analogues inhibited the high-affinity [^{14}C]GABA uptake into native plasma membrane vesicle suspensions less than did guvacine. There was little difference in GABA uptake inhibition between the (−)[1*R*,9*S*] and the (+)[1*S*,9*R*]*nor* bicuculline enantiomers, which were both more effective than the bicuculline enantiomers. The (+)[1*S*,9*R*]bicuculline enantiomer was 3-fold more potent than the (−)[1*R*,9*S*]bicuculline enantiomer. On the other hand, (−)[1*R*,9*S*]*nor* bicuculline is only slightly effective as inhibitor of $GABA_A$ receptors (Kardos et al., 1996). As the inhibition of GABA uptake and that of $GABA_A$ receptor function have different structural prerequisites (Krogsgaard-Larsen et al., 1980, 1994; Kardos et al., 1984), the opposite effectiveness of norbicuculline and bicuculline enantiomers in these processes suggests differences in their binding conformations (Kardos et al., 1996) and between the binding sites of the uptake protein and the receptor.

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