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INHIBITION OF NEURONAL GABA UPTAKE AND GLIAL β-ALANINE UPTAKE BY SYNTHETIC GABA ANALOGUES

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Abstract—Thirty three synthetic analogues of GABA were tested for their ability to act as inhibitors of neuronal or glial uptake sites for GABA and β-alanine, using [^{3}H]GABA uptake by synaptosome preparations from rat cerebral cortex, and [^{3}H]β-alanine uptake by cortical slices as test systems for neuronal and glial uptake sites, respectively. The results confirm that both uptake processes favour a substantially folded conformation. Neuronal uptake sites were significantly inhibited by analogues with long alkyl side chain substituents, such as dodecyl GABA-amide which was of comparable potency to GABA.

The inactivation of synaptically released GABA in mammalian CNS is thought to be mediated by high affinity uptake sites present on GABA-ergic neurones and on glial cells [1–3]. The development of specific inhibitors of the neuronal and/or glial uptake mechanism might offer an alternative approach to the use of GABA receptor agonists for modulating the effects of GABA in CNS by prolonging the duration of action of synaptically released GABA. A number of studies of GABA analogues as inhibitors of neuronal uptake have shown that the conformational requirements for uptake sites are quite different from the requirements for binding to postsynaptic GABA receptors [4–8]. The uptake process appears to involve a substantially folded conformation whereas a partially extended, eclipsed conformation is optimal for receptor interaction. The structural specificity of glial uptake sites has received much less attention, although these are probably also of significance in the metabolic disposition of GABA in CNS [3, 9, 10]. This paper presents the activities as inhibitors of neuronal and glial GABA uptake of a number of synthetic GABA analogues that we have also tested as inhibitors of GABA binding [11, 12].

Effects of test substances on neuronal uptake sites for GABA were assessed by measuring inhibition of [^{3}H]GABA uptake in homogenates of rat brain. Previous studies have shown that [^{3}H]GABA is predominantly accumulated by neuronal uptake sites in GABA-ergic synaptosomes under these conditions [3, 13]. The effects of GABA analogues on glial uptake sites were assessed by measuring inhibition of [^{3}H]β-alanine uptake by slices of rat brain. Under these conditions [^{3}H]β-alanine acts as a selective substrate for high affinity glial uptake sites for which GABA competes with a relatively high affinity [14]. Although it has been suggested that the uptake of [^{3}H]β-alanine may be mediated in part by taurine transport sites [9], we did not find taurine to be an effective inhibitor of [^{3}H]β-alanine uptake even when tested at high concentrations (1 mM).

MATERIALS AND METHODS

Synthetic methods

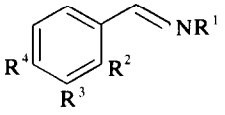
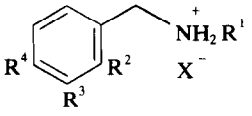
The syntheses of GABA derivatives used have been described previously [11, 12]. Analytical data for hydroxybenzylamines not described previously are presented in Table 1.

Dodecylamine was obtained from British Drug Houses (Poole, U.K.), urocanic acid and taurine were obtained from Aldrich (Gillingham, U.K.) and sodium tetradecyl sulphonate was kindly provided by the late Dr. A. J. Hyde (University of Strathclyde). Racemic substituted 4-aminobutanoic acids were prepared by published methods [15–17]. Aminoethane sulphonamides were prepared by the method of Winterbottom [18].

Biochemical methods

Neuronal uptake of GABA. [^{3}H]GABA uptake was measured in homogenates of rat cerebral cortex by a modification of the method described by Iversen and Johnston [4]. Samples of a 1:2 (v/v) 0.32 M sucrose homogenate, after removal of low speed particulate debris (1000 *g* for 10 min) equivalent to 2.5 mg original tissue were incubated in 1 ml Krebs bicarbonate solution at 25°. After a 5 min pre-incubation period, [^{3}H]GABA (2,3-[^{3}H]GABA; sp. act. 57 Ci/mmole, Radiochemical Centre, Amersham, U.K.), diluted with non-radioactive GABA, was added to give a final concentration of 0.05 μCi/ml and 0.1 μM. Incubation continued for 10 min at 25° and the tissue was recovered by centrifugation, using a Beckman Microfuge. The tissue pellet was resuspended in 0.2 ml distilled water and transferred to a counting vial for liquid scintillation counting, after addition of 4 ml ethoxyethanol and 10 ml toluene scintillant. Blanks were defined by subtracting counts obtained after incubation in the presence of a large excess (1 mM) of unlabelled GABA, and these represented approximately 5% of the normal control values. The tissue/medium ratio attained in control incubations was usually in the range 90–110:1. Test

Table 1. Chemical analytical results for hydroxybenzylamines

Compound	Per cent required	Found	^{1}H n.m.r. δ
			
R^1 R^2 R^3 R^4			
C_8H_{17} H OH H	C, 77.3	77.0	($CDCl_3$), 0.6–1.9, 15H, m;
m.p. 77–78°	H, 9.9	9.6	3.54,2H, t; 6.7–7.4,4H, m;
	N, 6.0	6.1	7.99,1H,s; 8.66,1H, s.
C_8H_{17} H OH OH	C, 72.3	72.5	(d_6-DMSO)0.83,3H, t;
m.p. 90–94°	H, 9.2	9.4	1.1–1.7,12H,m;3.38,2H,
	N, 5.6	5.6	t; 6.62,1H,d J = 7 Hz;
			6.84,1H,dd J = 7 and 3 Hz;
			7.07,1H, d J = 3 Hz; 7.91,
			1H, s.
C_8H_{17} OH OH H	C, 72.3	72.1	($CDCl_3$),0.86,3H, t;
m.p. 42–44°	H, 9.2	9.4	1.1–1.9,12H,m; 3.45,2H,
	N, 5.6	5.9	t; 6.3–6.9, 3H, m;
			7.92, 1H, s, 9.60–10, 2H, bd.
C_8H_{17} H OH OMe	C, 73.0	72.9	($CDCl_3$), 0.85, 3H, t;
m.p. 69–70°	H, 9.5	9.6	1.1–1.8, 12H,m; 3.47,
	N, 5.3	5.3	2H, t; 3.73, 3H, s;
			6.6–7.2, 3H, m; 7.9,
			1H, s.
			
R^1 R^2 R^3 R^4 X^-			
C_8H_{17} H OH H –	C, 76.6	76.5	($CDCl_3$) 0.7–1.7,15H,m;
(29)	H, 10.6	10.7	2.63,2H, t; 6.5–7.35,
m.p. 88–89°	N, 5.5	5.6	4H,m; 3.65,2H,s.
C_8H_{17} H OH OH Tos	C, 59.9	59.6	(d_6-DMSO) 0.83, 3H, t;
(31)	H, 7.5	7.8	1.0–1.7, 12H, m; 2.24,
m.p. 100–102°	N, 3.2	3.2	3H, s; 3.77, 2H, t;
	S, 7.3	7.3	3.96, 2H, s; 6.73, 3H, m;
	monohydrate		7.10, 7.45, 4H, ABq.
C_8H_{17} OH OH H Tos	C, 62.4	62.2	(d_6-DMSO), 0.84, 3H, t;
(33)	H, 7.8	8.0	1.0–0.7, 12H; 2.24, 3H, s;
m.p. 126–127°	N, 3.3	3.5	2.80,2H,t; 3.98,2H, s;
	S, 7.8	7.6	6.67,3H, m; 7.00, 7.20
			4H, ABq.
C_8H_{17} H OH OMe ClO_4	C, 52.5	52.5	(CD_3OD) 0.87, 3H, t;
(34)	H, 7.7	8.0	1.1–1.8, 12H, m; 2.96,
m.p. 103–105°	N, 3.8	4.2	2H, t; 3.78, 3H, s; 3.98,
	Cl, 9.7	9.7	2H, s; 6.82, 3H, s.

drugs were added 5 min before the addition of [^{3}H]GABA, and all incubations were performed in triplicate.

Glial uptake sites. [^{3}H]β-alanine uptake was measured in small slices of rat cerebral cortex as described by Schon and Kelly [14]. Aliquots of a cortical slice suspension, equivalent to 10 mg original tissue wet weight, were incubated with shaking in 5 ml Krebs phosphate solution for 10 min at 25° before addition of [^{3}H]β-alanine(β-2,3-[^{3}H]alanine; sp. act. 32.0 Ci/mmole; Radiochemical Centre, Amersham, U.K.) (final concentration 0.01 μM). Incubation was continued for 20 min and the slices harvested by filtration on Whatman No. 1 filter discs and washing with 2 × 5 ml Krebs solution. Blank values were defined by incubations performed at 0°, and amounted to 10–20% of control values. The tissue/medium ratio attained in control incubations was usually in the range 2.0–3.0:1. All incubations were performed in triplicate.

Test substances were assessed initially at a concentration of 1 mM, those causing more than 50% inhibition of [^{3}H]GABA or [^{3}H]β-alanine uptake were re-examined at a series of 3–6 lower concen-

trations and IC_{50} values (concentration of test substance needed to cause 50% inhibition of control uptake) were determined graphically by log-probit analysis.

RESULTS AND DISCUSSION

Neuronal uptake for GABA

For a zwitterionic neurotransmitter to pass through a non-polar membrane, it would be advantageous for the carrier to bring opposite charges in the transmitter close together thereby causing an intramolecular charge neutralisation [19]. Many studies of GABA analogues of restricted conformation indicate that this pattern of behaviour applies to neuronal GABA uptake. This is convincingly illustrated by the fact that 3-substituted piperidine carboxylic acids, in which the CO_2^- and NH_3^+ can fold towards each other, inhibit uptake, but the 4-isomers in which charged groups remain further apart, inhibit binding of GABA to postsynaptic membranes (Fig. 1); *cis*-cycloalkane amino acids are uptake inhibitors but the trans-isomers inhibit binding [7, 20]. Our initial aim in studying neuronal GABA uptake was to see whether this conformational concept could be refined further with the aid of the simple alkyl-GABA derivatives that we had prepared for GABA-binding studies. Table 2 lists the results for these and 20 other compounds studied.

When compared with the results we obtained for inhibition of [^{3}H]GABA-binding to postsynaptic membranes by alkyl-GABA derivatives [11] it is apparent that the neuronal uptake system is also sensitive to substitution in the GABA molecule, but the trends for uptake and receptor binding differ. Whereas for binding, 2-methylation caused a significantly greater reduction in binding potency than either 4- and 3-methylation, in the case of uptake, methyl groups on either 2- and 4-carbon atoms (2, 4) cause a greater loss in activity than at the 3-position (3); 2,4-dimethylation virtually abolishes activity. These results are consistent with the requirement for a folded conformation for uptake and two effects can be considered to be operating. Firstly, intramolecular steric interactions will greatly raise the energy of the folded conformation, especially where disubstitution occurs. Secondly, uptake may require the slimmest molecule possible, and branching methyl groups would thus be unfavourable: the zero activity of the 3-phenyl compound (7) supports this argument.

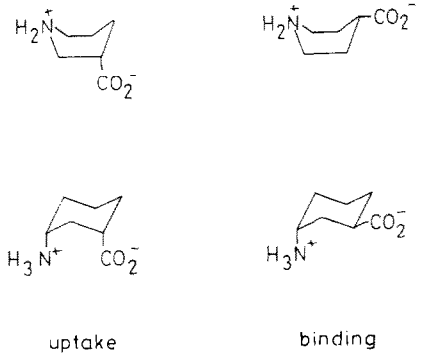


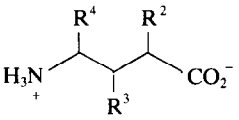
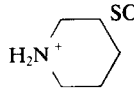
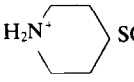
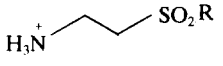
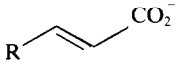
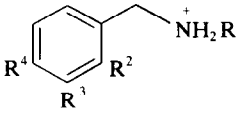
Fig. 1. Preferred conformations for inhibition of GABA uptake or binding to postsynaptic receptor sites are illustrated by 3-amino piperidine carboxylic acid, which inhibits GABA uptake, and 4-amino piperidine carboxylic acid which inhibits receptor binding.

Beart, Johnston and Uhr [5], and Johnston *et al.* [7] investigated a series of derivatives of 4-aminobut-2-enoic acid (4-amino-crotonic acid), which is a good inhibitor of GABA uptake (IC_{50} = 24 μM) [7]. Their results also show the weakening influences of substituents, both methyl groups and halogens. Two of our compounds (18, 19) are 4-aminocrotonic acid analogues that exist largely in a planar extended conformation so that overlap between the alkene and aromatic π-electron systems is maximised. It is not surprising, therefore, that these compounds are not inhibitors of GABA uptake. Results from Krogsgaard–Larsen's group [21] show similar features.

A striking feature of our results is that the sulphonic acids (9–13), so potent in binding inhibition, are such weak inhibitors of uptake, although piperidine-3-sulphonic acid (11) is more active than its 4-isomer (12) as would be expected from the above conformational considerations. However, the remaining compounds are conformationally unrestricted and it would be surprising if the greater bulk of the sulphonate group compared with carboxylate was alone responsible for the very weak activity. A possible explanation lies in the fact that sulphonates are extremely weak bases and could only be protonated to give a non-ionic species by an acid equal or greater in strength than a sulphonic acid. In biological systems, such acids are always fully ionised and therefore the sulphonic acid analogues of GABA also will be permanently ionised. On the other hand, a carboxylic acid can be protonated by a protein and in this way, a charge which would inhibit membrane transport can be neutralised. If such an event is essential for the uptake process, then the low activity of sulphonic acids can be understood. Indeed it could be that protonation of the carboxylate of GABA triggers a conformational change that activates the uptake system.

Recalling that uptake requires the transport of a zwitterionic molecule through a non-polar membrane, we were interested to see whether a long alkyl chain could potentiate uptake inhibition as it had with binding. Accordingly, two detergents (20, 21) and GABA-dodecylamide (22) were tested and considerable inhibitory activity was observed in each case. 2-Hydroxybenzylamines are slim molecules in which a closed conformation is forced upon the molecule. The amino and hydroxyl groups can thus act as recognition elements for the uptake system and a long alkyl chain could provide the potency. These effects can be seen in operation in compounds (24–27). Greatest activity was observed with a dodecyl side chain (24), which was of comparable potency to GABA itself but zero activity was observed with a butyl (26). The essential contribution of the hydroxyl group is shown by the fact that the 3-hydroxy compounds (28, 29) that have weak binding activity are very weakly active in uptake inhibition. With one exception, compound (33), all the

Table 2. Inhibition of [^{3}H]GABA uptake and [^{3}H]β-alanine uptake by synthetic analogues

Compound	GABA-uptake IC_{50} (μM)	GABA-uptake Per cent inhibition at 10^{-3} M	β-Alanine uptake IC_{50} (μM)	β-Alanine uptake Per cent inhibition at 10^{-3} M
				
(1) R^2=R^3=R^4=H	20	–	32	–
(2) R^2=Me R^3=R^4=H	–	70	–	0
(3) R^2=R^4=H R^3=Me	44	–	200	–
(4) R^4=Me R^2=R^3=H	105	–	–	75
(5) R^4=Me_2 R^2=R^3=H	–	42	–	0
(6) R^2=R^4=Me R^3=H	–	6	–	0
(7) R^2=R^4=H R^3=Ph	–	0	–	22
(8) $C_4H_9\overset{+}{N}H_2(CH_2)_3CO_2^-$	–	19	–	–
(9) $H_3\overset{+}{N}(CH_2)_3SO_3^-$	–	46	900	–
(10) $H_3\overset{+}{N}CH_2COCH_2SO_3^-$	–	17	–	–
(11) 	–	52	–	23
(12) 	–	18	–	30
				
(13) R=O^-	–	5	–	9.9
(14) R=NHPh	–	21	–	71
(15) R=$NHpC_6H_4NO_2$	–	39	–	89
(16) R=NHC_4H_9	–	17	–	21
(17) $H_3\overset{+}{N}(CH_2)_3SO_2NHpC_6H_4OMe$	–	–	–	20
				
(18) R=4-imidazolyl	–	8	–	12
(19) R=2-pyridyl	–	0	–	15
(20) $C_{12}H_{25}NH_2$	150	–	–	–
(21) $C_{14}H_{29}SO_3^-Na^+$	150	–	–	–
(22) $H_3N(CH_2)_3CONHC_{12}H_{25}$	27	–	–	44
(23) $H_3N(CH_2)_3CONHC_4H_9$	–	10	–	–
				
(24) R=$C_{12}H_{25}$ R^2=OH R^3=R^4 =H	25	–	–	47
(25) R=C_8H_{17} R^2=OH R^3=R^4=H	50	–	–	48
(26) R=C_4H_9 R^2=OH R^3=R^4=H	–	0	–	–
(27) R=CH_2Ph R^2=OH R^3=R^4=H	–	47	–	80
(28) R=$C_{12}H_{25}$ R^2=R^4=H R^3=OH	–	35	–	34
(29) R=C_8H_{17} R^2=R^4=H R^3=OH	–	17	–	71
(30) R=$C_{12}H_{25}$ R^2=H R^3=R^4=OH	500	–	1000	–
(31) R=C_8H_{17} R^2=H R^3=R^4=OH	–	39	–	48
(32) R=$C_{12}H_{25}$ R^2=R^3=OH R^4=H	–	9	–	22
(33) R=C_8H_{17} R^2=R^3=OH R^4=H	900	–	–	54
(34) R=C_8H_{17} R^2=H R^3=OH R^4=OMe	52	–	400	–

Results are expressed as per cent inhibition of [^{3}H]GABA or [^{3}H]β-alanine uptake when incubations were carried out with 1 mM test compound (mean of triplicate determinations), or as IC_{50} values (concentration of test compound causing 50% inhibition of uptake) determined graphically from results of testing 3–6 different concentrations of test substance.

polyhydroxyphenols studied (30–34) showed weaker activity than the 2-substituted compounds.

Taken together, our results are consistent with the notion that a compactly folded conformation is important for neuronal GABA uptake. Further, it would appear that slim molecules have some advantages, provided that they can attain the required conformation. The low activity of bicyclic nipecotic acid analogues found by Krogsgaard-Larsen [8] is consistent with this concept.

Glial uptake sites

Since both [^{3}H]GABA and [^{3}H]β-alanine uptake were measured at substrate concentrations well below those needed to saturate the respective uptake mechanisms, the inhibitory effects of test substances on these two uptakes are directly comparable. The ability of the glial uptake system to act upon β-alanine once again points to a compactly folded conformation for GABA in which charged groups are close together. Our results, listed in Table 2, are in accord with this concept. As with neuronal uptake, methyl groups at positions 2 and 4, singly (2, 4) or multiply (5, 6) cause a substantial reduction in the ability of the GABA analogues to inhibit β-alanine uptake. The least effect again is produced by methylation at the 3-position (3). Schousboe [10] has reported studies with cyclic analogues of GABA and has shown that *S*-β-proline and *R*-nipecotic acid act as inhibitors of glial uptake, again emphasizing the requirement for a folded conformation of GABA.

It is noticeable once again that sulphonic acids with strong inhibitory properties for GABA binding (9–12) do not interact strongly with the glial uptake system. Further, the short chain analogue taurine (13), which is the congener of β-alanine, is a very weak inhibitor of glial uptake indeed. This observation reinforces the suggestions made concerning protonation of carboxylate as a requirement for neuronal uptake and indicates that the glial system is similar in this respect. If these ideas are correct, then inhibition of uptake will be facilitated for analogues with pK_a's close to or a little weaker than GABA itself. Some sulphonamide derivatives of taurine (14–17) indicate such a trend. Sulphonamides typically have pK_a's of 9–10 but the introduction of anion stabilising groups can decrease the pK_a to as low as 5. Our results show that the most acidic sulphonamide bearing the acid-strengthening nitrophenyl substituent (15) is more active than the phenyl (14), and both are much more active than the butyl derivative (16) in which stabilisation of the anion by an aromatic ring is not possible. Significantly, the aminopropane sulphonamide (17) was as weakly active as the aminoethane sulphonamide (16), even though the former possesses an aromatic ring.

We found that neuronal GABA uptake was particularly sensitive to the presence of long alkyl chains [12]. Testing the same series of phenolic compounds (24–34) in the β-alanine uptake system revealed distinct differences from the neuronal system. In the β-alanine system, the most active compound contained a C-7 substituent (27) and the C-12 compound (24) that was most active as an inhibitor of neuronal uptake, was not a potent inhibitor of β-alanine uptake. Dodecyl compounds, including the GABA amide (22) show uniformly weaker activities (30–50% at 10^{-3}M) compared with the benzyl (27) and octyl derivatives (25, 29). This could be an indication that the glial uptake carrier molecule has a shallower hydrophobic region than the corresponding neuronal system.

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