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PHARMACOLOGICAL SPECIFICITY OF SYNAPTOSOMAL AND SYNAPTIC MEMBRANE γ-AMINOBUTYRIC ACID (GABA) TRANSPORT PROCESSES*

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Abstract—Amino cyclopentane and amino cyclohexane carboxylic acids were examined as potential inhibitors of γ-aminobutyric acid (GABA) uptake in brain synaptosomes and in synaptic membrane vesicles. The resealed synaptic plasma membrane vesicle preparation was used extensively in determining the potency inhibition of GABA uptake by these agents and in comparing their activities to the activities of various acyclic GABA analogs and to analogs of piperidyl-3-carboxylic acid (nipecotic acid). A number of the cyclic and acyclic GABA analogs also stimulated the carrier-mediated efflux of [^{3}H]GABA from preloaded synaptic membrane vesicles, whereas nipecotic acid did not increase the efflux of [^{3}H]GABA in a dose-dependent manner. These results suggest that there is a competitive type of interaction of the cyclic and acyclic GABA analogs with membrane uptake carriers and that a more complex type of action of nipecotic acid on these carriers occurs. The order of potency of uptake inhibition by some of these agents was compared with previously published orders of activation of physiologic GABA receptors by these compounds.

A Na^+-dependent, high affinity transport system for γ-aminobutyric acid (GABA) in neurons and in glia cells of the central nervous system is an important mechanism for the termination of the action of this putative neurotransmitter at the synapse [1]. Some of the biochemical and pharmacological aspects of this uptake process in synaptosomes and brain slices have been well characterized [2–4]. On the basis of the information available from these studies it appears that the GABA uptake carrier in neuronal membranes possesses high structural specificity [2–6].

The present study of the neuronal transport system represents a further exploration of the topography of the uptake carrier site through the use of cyclohexane and cylopentane amino carboxylic acid analogs of GABA, as well as through the use of other GABA analogs and membrane-active agents. Some of the compounds employed in this study have been examined previously by Hitzemann and Loh [3] for their effects on the [^{3}H]GABA uptake activity of rat brain synaptosomes. However, even though some of the cyclopentane and cyclohexane amino carboxylic acids tested in that study were found to be potent inhibitors of [^{3}H]GABA uptake, some important comparisons were not made. For example, *cis*-3-amino cyclohexane carboxylic acid (*cis*-3-ACHC) was one of the most potent uptake inhibitors and yet its isomer *trans*-3-ACHC was not tested in that system. Similarly, although *trans*-2-amino cyclopentane carboxylic acid (*trans*-2-ACPC) had moderate inhibitory activity on the uptake system, no information was provided about the actions of *cis*-2-ACPC. Finally the inhibition of GABA transport that was caused by these agents was not compared to that of other known potent GABA uptake blockers such as piperidyl-3-carboxylic acid (nipecotic acid) [7] and its analogs. The availability of such information would be quite helpful in attempting to determine the topography of the active site of the GABA carrier system.

An additional issue, addressed in the present study, is the distinction between the activity of various GABA analogs on the GABA transport system in general and their actions on the uptake of [^{3}H]GABA via a homoexchange process. It has been suggested previously that what may appear to be carrier-mediated transport of GABA in synaptosomes may, in fact, be uptake of [^{3}H]GABA brought about by homoexchange with intrasynaptosomal GABA [8–10]. This possibility was clearly recognized by Hitzemann and Loh [3] and led these investigators to confine their description to the activity of the GABA analogs to the uptake of [^{3}H]GABA rather than to extend their findings to the GABA transport system of neurons in general. It was recently shown, however, that synaptosomal preparations have a net GABA uptake that is distinguishable from homoexchange processes [11]. To address this ambiguity about transport versus homoexchange activity, we have compared the synaptosomal [^{3}H]GABA uptake activity, and its inhibition by various agents, to the uptake process of purified resealed synaptic plasma membrane vesicles that are presumably free of intravesicular GABA. Though an initial biochemical and biophysical characterization of the GABA uptake system in the resealed synaptic plasma membrane vesicles has been done

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by Kanner [12], we were primarily interested in determining whether these synaptic membrane vesicles retain the same pharmacological and structural specificity of the GABA uptake carrier that is observed in the synaptosomal preparations. In addition, it was felt that these membrane preparations might have the distinct advantage of not being as greatly contaminated with the enzymes involved in GABA metabolism as the crude synaptosomal preparations are, and that they may provide a means for studying the effects of GABA analogs not only on carrier-mediated uptake, but also on carrier-mediated GABA efflux.

MATERIALS AND METHODS

Preparation of the crude synaptosomal fraction. All tissue preparations were obtained from adult male Sprague–Dawley rats killed by cervical dislocation. All steps in the tissue preparation were conducted at 0–4°. Brain homogenates in 0.32 M sucrose were centrifuged at 1900 $g \times 5$ min to remove nuclear and cell debris material. The supernatant fraction was subsequently centrifuged at 17,000 $g \times 10$ min to obtain mitochondrial pellet, which is known to contain intact nerve endings [13]. The crude mitochondrial fraction was washed by resuspending it in 6 ml of Krebs–Henseleit buffer (132 mM NaCl, 5 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 10 mM glucose, 50 mM Tris–HCl, pH 7.4) and by centrifuging it at 8000 $g \times 10$ min. The final pellet was resuspended in 4 ml of Krebs–Henseleit buffer.

Preparation of the synaptic membranes. The crude mitochondrial fraction was resuspended in 0.32 M sucrose, layered on a ficoll–sucrose discontinuous gradient (7.5 and 13% ficoll in 0.32 M sucrose) and centrifuged at 63,500 $g \times 45$ min. The synaptosome-enriched subfraction was obtained by retrieval of the particulate material which bands at the interface of 7.5 and 13% ficoll [14].

The synaptosomal fraction was diluted 10-fold with 0.32 M sucrose and centrifuged at 39,000 $g \times 25$ min. The pellet was resuspended in a large volume of hypnotic medium (5 mM Tris–HCl, 1 mM K–EDTA, pH 7.4) and stirred at 4° for 45 min. Following this step of osmotic rupturing, the suspension was centrifuged at 20,000 $g \times 15$ min. The synaptic membrane pellet was handled according to the method of Kanner [12], which involved its resuspension in a solution of 0.32 M sucrose, 5 mM Tris–SO_4, 1 mM $MgSO_4$, 0.5 mM EDTA, pH 7.4, at a final protein concentration of 6–12 mg/ml. The membrane suspension was divided into appropriate aliquots and quickly frozen in liquid N_2 and stored at −80°. The frozen synaptic membrane preparations retained their Na^+-dependent, high affinity GABA uptake activity for up to 2 months after the initial freezing.

Reconstitution of the frozen synaptic membranes. The appropriate amount of 0.1 M potassium phosphate buffer–1 mM $MgSO_4$ buffer (pH 7.4) was added to the frozen synaptic membranes to dilute them to a protein concentration of 2 mg/ml. The membranes were incubated for 5 min at 37° and allowed to cool to room temperature (23–25°) prior to use in the uptake assays. These membrane suspensions were fully active even after 24 hr of storage at 4°.

Uptake assays. All assays of [^{3}H]GABA uptake by synaptosomes were initiated by the addition of 100 μl of synaptosomal suspension (2 mg/ml) to 400 μl of Krebs–Henseleit buffer, which contained 0.4 μCi [^{3}H]GABA and variable amounts of unlabeled GABA or test compound. All samples were incubated at 37° for 1 min (except for the time kinetic experiments). The incubations were terminated by adding 1 ml of ice-cold Krebs-Henseleit buffer and by filtering immediately through Whatman GF/B glass fiber filters. The filters were washed with 5 ml of ice-cold Krebs–Henseleit buffer, dried, and the radioactivity associated with each filter was measured by scintillation spectrometry following the addition of Triton X-100–toluene (1:3, v/v) based scintillation fluid. Blanks were prepared by substituting Krebs–Henseleit buffer for the synaptosomal suspension. These buffer blanks were found to be equivalent to synaptosomal samples incubated in Na^+-free Krebs–Henseleit buffer (NaCl replaced by choline chloride).

The uptake of [^{3}H]GABA into synaptic membrane vesicles was initiated by the addition of 20 μl of the vesicle suspension (2–3 mg/ml) to 180 μl of incubation medium containing 0.4 μCi of [^{3}H]GABA, variable amounts of unlabeled GABA, and the compound being tested, in 154 mM NaCl–1 mM $MgSO_4$ solution. All incubations were carried out at 24° for 5 min (except for the time and substrate concentration kinetic experiments), and the reactions were stopped by the addition of 2 ml of ice-cold NaCl–$MgSO_4$ solution, followed by filtration of the contents of each tube through 0.45 μm Millipore filters. The filters were washed with 2 ml of ice-cold NaCl–$MgSO_4$ solution and dried at 90°. Blanks were prepared by substituting the NaCl–$MgSO_4$ solution for the membrane suspension. The buffer blanks retained as much radioactivity on the filters as did membrane samples that were incubated in KCl–$MgSO_4$ solution. Each filter was dissolved in 2-ethoxyethanol, and the associated radioactivity was determined by scintillation spectrometry in a 2-ethoxyethanol–toluene (1:5, v/v) based scintillation fluid.

Carrier-mediated exchange assay. The uptake component of each assay was initiated by the addition of 20 μl of synaptic membrane vesicle suspension (2–3 mg/ml) to 180 μl of an incubation medium that contained 0.4 μCi [^{3}H]GABA and 10^{-7} M GABA in a 154 mM NaCl–1 mM $MgSO_4$ solution. All incubations were carried out at 23–25° for 5 min. Efflux was initiated at the end of the 5-min incubation by the addition of 5 ml of the NaCl–$MgSO_4$ solution or of the same solution that contained the compounds being tested. The efflux phase was terminated by filtering the contents of each tube and washing the filters with 2 ml of ice-cold NaCl–$MgSO_4$ solution. The filters were processed for scintillation counting as described in the uptake assay procedure. When *p*-chloromercuribenzenesulfonic acid (PCMS) was used (100 μM), it was always added after the uptake component of the assay was complete. The membrane vesicles, preloaded with [^{3}H]GABA, were

incubated at 23–25° for an additional 5 min in the presence of 100 μM PCMS, diluted with NaCl–$MgSO_4$ or with the same solution that contained the test compound, and incubated further as described above for the efflux phase. Membrane vesicles that were lysed in distilled water during the loading step functioned as blanks for these studies.

Dissolution of the compounds. All compounds were dissolved in distilled water, except for the following: diazepam, *N*-desmethyldiazepam, and oxazepam were dissolved in dimethylsulfoxide, and quinacrine was dissolved in 0.125 N HCl.

Protein determination. The protein concentration of all samples was determined by the method of Lowry *et al.* [15].

Materials. [^{3}H]-γ-Aminobutyric acid (36.12 Ci/mmole) was obtained from the New England Nuclear Corp., Boston, MA. Tetracaine hydrochloride, 4-amino-3-hydroxybutanoic acid, quinacrine, aminooxyacetic acid, 4-aminobutanoic acid, PCMS, *p*-hydroxymercuribenzoic acid, and Triton X-100 were purchased from the Sigma Chemical Co., St. Louis, MO. 3-Guanidinopropanoic acid was obtained from CalBiochem, La Jolla, CA. All other compounds were synthesized in our laboratories. Diazepam, *N*-desmethyldiazepam and oxazepam were supplied by Dr. V. Stella (Department of Pharmaceutical Chemistry, University of Kansas).

Data analysis. All IC_{50} values were determined from log-probit analysis. The K_i of each inhibitor was obtained by using the relationship: $K_i = IC_{50} \cdot K_D/([GABA] + K_D)$. The retention coefficient in efflux studies was defined as the ratio of the amount of [^{3}H]GABA retained by the test sample to the amount of [^{3}H]GABA retained by control samples filtered immediately after the loading step (no efflux condition). The differences in the inhibitory activity of the grouped data for each compound tested were analyzed for significance by analysis of variance.

RESULTS

GABA uptake by the crude synaptosomal fraction. Uptake of [^{3}H]GABA by the synaptosomes was linear over the range of protein concentrations tested (0.2 to 1.2 mg/ml, final concentration in the assay). Time kinetic studies showed that synaptosomal GABA uptake was linear up to 1 min. This uptake process was dependent on the concentration of GABA in the external medium and exhibited a K_m of 3.6 ± 0.6 μM and a V_{max} of 980 ± 110 pmoles · (mg protein)$^{-1}$·min^{-1}(mean ± S.E., N = 3). To estimate the contribution to the synaptosomal [^{3}H]GABA uptake by possible glial contaminants, the effects of β-alanine, a glial GABA uptake inhibitor [16], on the GABA transport activity were determined. β-Alanine at a concentration of 100 μM inhibited [^{3}H]GABA (0.12 μM) uptake by 17.0 ± 8.5 per cent (mean ± S.E., N = 3). On the basis of the above characteristics, the uptake system for GABA in this crude synaptosomal preparation was considered to be similar to that described previously for more purified synaptosomal preparations [3].

The activities of several amino cyclopentane and amino cyclohexane carboxylic acids as potential antagonists of the synaptosomal GABA uptake carrier were also examined (Table 1). Of the agents tested, the two most potent inhibitors of [^{3}H]GABA uptake in synaptosomes were *cis*-2-ACPC (K_i = 60.3 μM) and *cis*-3-ACHC (K_i = 71.7 μM). The *trans*-isomer of 2-ACPC was found to be only half as potent as the *cis*-isomer, whereas the K_I of *trans*-3-ACHC was approximately twenty times higher than that of its *cis*-isomer (Table 1).

Table 1. Inhibition constants of ACPC and ACHC derivatives for the inhibition of [^{3}H]GABA uptake in synaptosomes*

Compound	IC_{50} (μM)	K_i (μM)
cis-2-ACPC	63.1	60.3
trans-2-ACPC	119.0	114.0
cis-3-ACHC	75.0	71.7
trans-3-ACHC	1330.0	1300.0
cis-4-ACHC	398.0	381.0
cis-2-ACHC	> 1000.0	> 1000.0

* Rat brain synaptosomes were prepared and assayed for [^{3}H]GABA uptake activity as described in Materials and Methods. Each value is the mean of four to eight determinations from two to four synaptosomal preparations.

GABA uptake in synaptic plasma membrane vesicles. Time kinetic studies of [^{3}H]GABA (0.156 μM) uptake by synaptic plasma membrane vesicles were linear for approximately 1 min, reaching equilibrium in 5 min. Analysis of the concentration-dependent kinetics of GABA uptake by these membranes (initial velocity of uptake was measured at 30 sec) revealed the presence of one apparent transport process with the following kinetic characteristics as determined by the least squares line-fitting of Lineweaver–Burk plots: K_m = 2.15 μM and V_{max} = 57 pmoles · (mg protein)$^{-1}$ · min^{-1}. Maximum activation of this uptake was obtained in the presence of Na^+ concentration gradient ([Na^+] out > [Na^+] in). Synaptic plasma membrane vesicles that were internally loaded with 154 mM NaCl–1 mM $MgSO_4$ and were incubated in the same medium (no Na^+ gradient) accumulated 1.64 pmoles GABA · (mg protein)$^{-1}$ · (5 min)$^{-1}$, whereas vesicles that were internally loaded with 0.1 M potassium phosphate buffer–1 mM $MgSO_4$ (inward-directed Na^+ gradient) took up 7.3 pmoles GABA · (mg protein)$^{-1}$ · (5 min)$^{-1}$. Membranes that were internally loaded with 0.1 M sodium phosphate buffer–1 mM $MgSO_4$ accumulated amounts of [^{3}H]GABA approximately equal to those of the NaCl–$MgSO_4$ loaded membranes. These results were indicative of the dependence of the concentrative GABA uptake process on an existing [Na^+] gradient (out > in) with only a small contribution to GABA accumulation from passive GABA flux or from Na^+-dependent GABA binding to the synaptic membranes. Further evidence for the transport of GABA into the intravesicular space in these membranes was the observation that increases in the extravesicular osmolarity of the incubation medium, brought about by the addition of either 0.16 or 0.32 M sucrose to the 154 mM NaCl–1 mM $MgSO_4$ incubation buffer, led to

substantial reductions of GABA accumulation in these vesicles. The mean (± S.E.) GABA uptake in the $NaCl$–$MgSO_4$ medium was 7.5 ± 0.1 pmoles · (mg protein)$^{-1}$ · (5 min)$^{-1}$ (N = 3), whereas in the presence of added 0.16 or 0.32 M sucrose the uptake decreased to 6.0 ± 0.2 and 4.2 ± 0.4 pmoles · mg^{-1} · (5 min)$^{-1}$ (N = 3) respectively. The increases in extravesicular osmolarity would be expected to bring about a decrease in the intravesicular volume which would thus lead to diminution of the GABA-accumulating capacity of these vesicles. These observations are identical to those described previously by Kanner [12].

A determination of the sensitivity of this uptake process to exposure of the membranes to two organomercurials revealed a difference between the uptake-inhibiting potency of *p*-hydroxymercuribenzoic acid (PHMB) and that of *p*-chloromercuribenzenesulfonic acid (PCMS). At a concentration of 500 μM and with no preincubation period, PCMS inhibited [^{3}H]GABA uptake by 67.9 ± 1.1 per cent (mean ± S.E., N = 3), whereas PHMB inhibited GABA uptake by 97.5 ± 3.5 per cent (N = 6). Preincubation of the membranes for 5 min with 100 μM PCMS caused a level of inhibition of [^{3}H]GABA uptake (68.2 per cent, N = 2) that was similar to the level produced by 500 μM PCMS introduced directly in the uptake assay medium. The potent inhibition of the membrane-vesicle GABA transport process caused by PHMB has been reported previously also by Kanner [12].

In addition to the agents that inhibited the uptake process in the synaptosomal fraction, several other cyclopentane and cyclohexane carboxylic acids also were tested for inhibition of the GABA transport system in brain synaptic membrane preparations; their effects on that process are shown in Table 2. Included in the table are data of the inhibitory effects produced by piperidyl-3-carboxylic acid (nipecotic acid) and by some of its analogs, by acyclic GABA analogs, and by some membrane-active agents. Some of these membrane-active agents, such as the benzodiazepines, have been known to block GABA uptake by synaptosomes [17]. As is evident from the data presented in Table 2, the synaptosomal GABA uptake blockers *cis*- and *trans*-2-ACPC and *cis*-3-ACHC were also potent inhibitors of GABA uptake by the synaptic membrane vesicles, as were the *cis*-

Table 2. Effects of various GABA analogs and membrane active compounds on GABA uptake activity by synaptic membrane vesicles*

Agent	% Inhibition of GABA uptake
Cyclic GABA analogs	
trans-3-ACPC	100.0 ± 0.6
cis-3-ACPC	96.2 ± 1.6
cis-2-ACPC	92.0 ± 4.0
trans-2-ACPC	95.5 ± 1.5
cis-3-ACHC	97.0 ± 1.0
trans-3-ACHC	29.7 ± 4.8
cis-4-ACHC	4.2 ± 4.0
trans-4-ACHC	5.6 ± 4.6
cis-2-ACHC	21.5 ± 3.2
trans-2-ACHC	19.0 ± 3.2
cis-3-Aminomethylcyclopentane carboxylic acid	42.4 ± 10.0
Piperidyl-3-carboxylic acid	100.0 ± 1.5
3-Piperidyl acetic acid	91.3 ± 0.4
Piperidyl-4-carboxylic acid	52.7 ± 7.0
Acyclic GABA analogs	
3-Guanidinopropanoic acid	99.4 ± 2.2
4-Amino-3-hydroxybutanoic acid	98.5 ± 4.5
5-Aminopentanoic acid	98.6 ± 3.2
6-Aminohexanoic acid	18.8 ± 6.7
4-Hydroxybutanoic acid	34.1 ± 1.1
Amino-oxyacetic acid	38.3 ± 0.9
Membrane active agents	
Diazepam	98.6 ± 1.3
Desmethyldiazepam	100.0 ± 6.3
Oxazepam	70.2 ± 1.7
Tetracaine (1 mM)	92.9 ± 0.7
(0.1 mM)	2.6 ± 7.4
Quinacrine (1 mM)	100.0 ± 1.9
(0.1 mM)	56.8 ± 1.3

* Synaptic plasma membrane vesicles were prepared and assayed for [^{3}H]GABA uptake activity as described in Materials and Methods. The compounds were tested at a concentration of 1.0 mM unless otherwise indicated. Each value is the mean per cent inhibition (± S.E.) obtained from three to nine determinations (two to three membrane preparations). Uptake of [^{3}H]GABA by these membrane vesicles in the absence of any agents was 7.1 ± 0.3 pmoles · (mg protein)$^{-1}$ · min^{-1}.

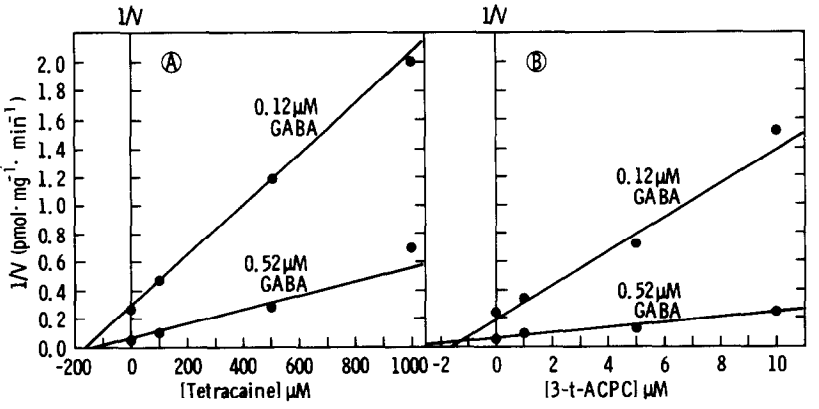

Fig. 1. Dixon plots of tetracaine (A) and of *trans*-3-ACPC (B) inhibition of GABA uptake in synaptic membrane vesicles. The uptake assays were conducted as detailed in Materials and Methods except that a 1-min incubation with 0.12 or 0.52 μM GABA was used. The K_i values of 171 μM for tetracaine and 1.4 μM for *trans*-3-APCP were calculated from least squares line-fitting of the experimental data. Each point is the mean of triplicate determinations from a single membrane preparation. The S.E.M. for each point was less than 10 per cent.

and *trans*-3-ACPC isomers, nipecotic acid and its analog 3-piperidyl acetic acid (Table 2). The benzodiazepines and the local anesthetic agents tetracaine and quinacrine also strongly inhibited the GABA uptake activity in these membrane vesicles (Table 2). Unlike some of the other potent inhibitors of the GABA transport system, however, the local anesthetics inhibited GABA uptake over a rather narrow range of concentrations (Table 2). Furthermore, the kinetics of inhibition produced by tetracaine were those of a non-competitive inhibitor of GABA uptake (Fig. 1A), whereas those of *trans*-3-ACPC were typical of a competitive inhibitor of [^{3}H]GABA uptake (Fig. 1B).

The constants of inhibition (IC_{50} and K_i) of the GABA transport system in synaptic plasma membrane vesicles were determined only for those GABA analogs that exhibited substantial inhibitory activity of GABA uptake when present at a 1 mM concentration (Table 2). These agents were tested over the concentration range of 1 μM to 1 mM (0.1 μM to 1 mM for *trans*-3-ACPC). It is apparent from the K_i values shown in Table 3 that the amino cyclopentane carboxylic acids *trans*-3-ACPC and *cis*-3-ACPC were considerably more potent as inhibitors of synaptic membrane GABA uptake activity than either *cis*- or *trans*-2-ACPC or *cis*-3-ACHC. The rank order of the inhibitory potency of the cyclopentane and cyclohexane derivatives tested, of nipecotic acid, and of 4-amino-3-hydroxybutanoic acid is shown below. Included are also the values of P obtained from statistical analysis of the differences between each agent and the preceeding agent(s): *trans*-3-ACPC ≃ nipecotic acid ($P > 0.05$) > *cis*-3-ACPC ($P < 0.01$) > *cis*-3-ACHC ($P < 0.05$) > 4-amino-3-OH-butanoic acid ($P < 0.05$) > *trans*-2-ACPC ($P < 0.05$) ≃ *cis*-2-ACPC ($P > 0.05$). It should also be noted that the K_i for the competitive inhibition of GABA uptake (K_i = 1.4 μM) was very similar to the K_i calculated for this agent on the basis of its IC_{50} value (K_i = 2.2 μM, Table 3).

Carrier-mediated efflux of preloaded [^{3}H]GABA. It was assumed that, if the cyclopentane and cyclohexane carboxylic acid analogs functioned as competitive inhibitors of the GABA transport carrier system, these compounds would be very effective in causing the efflux of preloaded [^{3}H]GABA from the synaptic membrane vesicles through carrier-mediated exchange. Such an effect of non-radioactively labeled GABA on the efflux of [^{3}H]GABA from synaptic membrane vesicles has already been described [12].

The retention coefficients, which represent the efflux-inducing potencies of the GABA analogs, together with the fractional inhibitions of GABA uptake produced by the same concentrations of these agents are shown in Table 4. On the basis of the data shown in this table, it is clear that the most potent blockers of GABA uptake activity had the lowest retention coefficients and the highest fractional inhibitions for the same concentration of the agent. The only exception to this trend was the set of values obtained for nipecotic acid. The introduction of this

Table 3. Inhibition constants of various GABA analogs for the inhibition of GABA uptake in synaptic membranes*

Compound	IC_{50} (μM)	K_i (μM)
trans-3-ACPC	2.4	2.2
Piperidyl-3-carboxylic acid	4.5	4.2
cis-3-ACPC	6.7	6.2
cis-3-ACHC	22.4	20.9
4-Amino-3-hydroxybutanoic acid	23.7	22.1
3-Guanidinopropanoic acid	26.6	24.8
trans-2-ACPC	33.5	31.2
3-Piperidyl acetic acid	37.6	35.1
5-Aminopentanoic acid	37.6	35.1
cis-2-ACPC	39.8	37.1

* Synaptic membranes were prepared and assayed for [^{3}H]GABA activity as described in Materials and Methods. Each value is the mean of three to nine determinations from one to three membrane preparations.

Table 4. Retention coefficient and fractional inhibition of GABA analogs and membrane-active agents*

Agent	Concn	Retention coefficient	Fractional Inhibition
GABA	10 μM	0.36	
trans-3-ACPC	10 μM	0.38	0.70
cis-3-ACPC	10 μM	0.55	0.55
Piperidyl-3-carboxylic acid	10 μM	0.65	0.66
Piperidyl-4-carboxylic acid	1 mM	0.49	0.53
3-Piperidyl acetic acid	100 μM	0.36	0.66
4-Amino-3-hydroxybutanoic acid	100 μM	0.20	0.77
5-Aminopentanoic acid	100 μM	0.30	0.70
3-Guanidinopropanoic acid	50 μM	0.37	0.65
Tetracaine	100 μM	1.48	0.03
Quinacrine	100 μM	1.57	0.57

* Synaptic membrane vesicles were prepared and assayed for [^{3}H]GABA uptake and efflux activity as described in Materials and Methods. All incubations for either uptake or efflux of GABA were carried out at 24° for 5 min. Each value is the mean of six to nine determinations from two to five membrane preparations. Uptake of [^{3}H]GABA by control membranes was 7.3 ± 0.3 pmoles · (mg protein)$^{-1}$ · (5 min)$^{-1}$ (mean ± S.E.). The amount of GABA retained by control membranes at 5 min incubation was 4.3 ± 0.4 pmoles/mg protein. Fractional inhibition = per cent inhibition of uptake/100. Retention coefficient = the ratio of [^{3}H]GABA retained by the test sample to that retained by control samples (see "Data analysis").

agent into the diluting solution at a concentration of 10 μM caused a moderately low efflux of [^{3}H]GABA (retention coefficient = 0.65) (Table 4). Because the retention coefficient represents the fraction of total [^{3}H]GABA found in the intravesicular space presumably following the activation of carrier-mediated efflux and because the fractional inhibition value represents the fraction of total GABA found in the extravesicular space following the inhibition of carrier-mediated uptake by the same concentration of the agent, the sum of the two values should equal 1. This correlation was found to hold true for all compounds considered to be structural GABA analogs (Table 4), except tor nipecotic acid (retention + inhibition = 1.31, Table 4). In this respect, the behaviour of nipecotic acid was similar to that exhibited by the local anesthetic agents quinacrine and tetracaine. Both local anesthetics had retention + inhibition values that were greater than 1 (2.14 and 1.51 respectively) and which were the result of lack of any [^{3}H]GABA efflux in the presence of these agents (Table 4).

Efflux of [^{3}H]GABA from the synaptic membrane vesicles was also observed in the absence of any of the agents shown in Table 4. The addition of the NaCl–$MgSO_4$ diluting solution to vesicles that were preloaded with [^{3}H]GABA caused a 5–10 per cent efflux at zero time, a 20–25 per cent efflux after 2.5 min of incubation, and a 30–35 per cent efflux after 5 min of incubation. The substitution of NaCl–$MgSO_4$ by a KCl–$CaCl_2$ mixture in the diluting medium did not alter the rate of spontaneous

Table 5. Effects of various GABA analogs on [^{3}H]GABA retention coefficients of synaptic membrane vesicles*

Compound	Retention coefficients		
	Efflux time 0 min	2.5 min	5 min
trans-3-ACPC	0.27 ± 0.00	0.25 ± 0.04	0.19 ± 0.00
cis-2-ACPC	0.41 ± 0.09	0.30 ± 0.00	0.30 ± 0.02
cis-3-ACHC	0.41 ± 0.08	0.38 ± 0.03	0.30 ± 0.04
cis-3-ACPC	0.68 ± 0.03	0.45 ± 0.01	0.38 ± 0.01
trans-3-ACHC	1.09 ± 0.03	1.11 ± 0.03	1.07 ± 0.04
Piperidyl acetic acid	0.69 ± 0.10	0.59 ± 0.04	0.39 ± 0.00

* Synaptic plasma membrane vesicles were prepared and assayed for [^{3}H]GABA efflux at various time intervals as described in Materials and Methods. Each compound was tested at a concentration of 100 μM except for *trans*- and *cis*-3-ACPC, which were tested at 10 μM. The data expressed as retention coefficient values. The efflux time represents the period of time during which we measured how much was retained. Each value is the mean ± S.E. of three determinations from a single membrane preparation. The amount of GABA retained by control membranes was 4.2 pmoles/mg protein.

[^{3}H]GABA efflux. A large, instantaneous (zero time) efflux was observed only when an active blocker of GABA uptake was present in the NaCl–$MgSO_4$ diluting solution. The time kinetic characteristics of this presumably carrier-mediated [^{3}H]GABA efflux are shown in Table 5. Considerable [^{3}H]GABA efflux had already occurred at time zero in the presence of all active compounds tested (Table 5). Zero time actually represented a time interval greater than 0 sec but less than 10 sec. This was the time period needed to add the diluting solution to the reaction vessel and to filter the entire contents through a 0.45 μm Millipore filter.

Further evidence that this very rapid efflux of preloaded [^{3}H]GABA was primarily due to a carrier-mediated process was provided by the observation that the addition of the organomercurial PCMS (100 μM) after loading of the membrane vesicles with [^{3}H]GABA led to an almost complete inhibition of the efflux-inducing action of *trans*-3-ACPC, *cis*-3-ACPC, and *cis*-3-ACHC (data not shown). Thus, both the carrier-mediated GABA uptake activity of these vesicles and the GABA efflux produced by the various GABA analogs were organomercurial-sensitive processes. In addition, the dose–response relationships of GABA uptake inhibition and analog-induced [^{3}H]GABA efflux for some of these GABA analogs, but not for nipecotic acid, were almost identical, as is shown in Fig. 2.

DISCUSSION

The development of specific inhibitors of the GABA uptake carrier of brain synaptic membranes could be important in the elucidation of the topographic characteristics of this site and in the delineation of the differences that exist between the GABA carrier, and the GABA receptor, sites. An analysis of the actions of cyclopentane and cyclohexane amino carboxylic acid analogs of GABA on the uptake system for this amino acid was pursued in intact synaptosomes and in purified resealed synaptic membranes.

The results obtained with the resealed synaptic membrane vesicles revealed the usefulness of these preparations in the study of the carrier-mediated GABA uptake process. This GABA transport system was strictly dependent on an established [Na^+] gradient (out > in) and exhibited kinetic characteristics similar to those of the synaptosomal uptake process. These findings confirm the observations reported previously by Kanner [12]. The two most effective inhibitors of this transport system were *trans*-3-ACPC and *cis*-3-ACPC (Table 3). Both of these cyclopentane amino acid derivatives were approximately three to ten times more effective than either *cis*-3-ACHC or the *trans*- and *cis*-isomers of 2-ACPC. The cyclic GABA analogs *trans*-3-ACHC, *cis*-2-ACHC, and *trans*-2-ACHC were very weak inhibitors of the GABA transport system, and the derivatives *trans*-4-ACHC and *cis*-4-ACHC were almost completely inactive in inhibiting the GABA uptake activity of these membrane preparations.

It has been shown previously in rat cerebral cortex slice preparations that [^{3}H]-*cis*-3-ACHC is transported into neurons by a relatively high affinity uptake process (K_m = 85.1 μM) [6]. This [^{3}H]-*cis*-3-ACHC uptake was strongly blocked by GABA and was inhibited to a lesser extent by β-alanine [6]. These observations would suggest that the cyclic GABA analog *cis*-3-ACHC can activate the membrane GABA uptake carriers. The demonstration in the present study that the most effective blockers of GABA uptake amongst the ACPC and ACHC

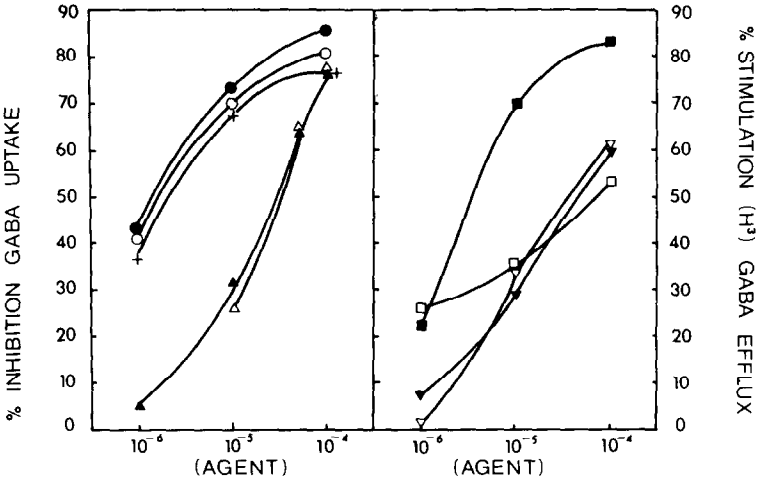

Fig. 2. Stimulation of GABA efflux from vesicles preloaded with [^{3}H]GABA and inhibition of GABA uptake into synaptic membrane vesicles. Both efflux and uptake studies were conducted at 24° for 5 min as described in Materials and Methods. Key: (○, △, ▽, □) efflux stimulation; and (●, ▲, ▼, ■) uptake inhibition for the following agents: *trans*-3-ACPC (○, ●), 4-amino-3-OH-butanoic acid (△, ▲), 3-piperidyl acetic acid (▽, ▼), and nipecotic acid (□, ■). Efflux of [^{3}H]GABA induced by unlabeled GABA is also shown (+). Each point is the mean of three to six determinations from two to five membrane preparations. Control [^{3}H]GABA uptake was 8.5 ± 0.2 pmoles · (mg protein)$^{-1}$ · (5 min)$^{-1}$ and [^{3}H]GABA retention by control membranes was 4.8 ± 0.4 pmoles/mg protein.

derivatives were also the most active in initiating the carrier-mediated efflux of [^{3}H]GABA from preloaded membrane vesicles provides additional support for the presumed direct interaction of these agents with the GABA carrier. Since the ACPC- and ACHC-stimulated [^{3}H]GABA efflux was inhibited by pretreatment of the membranes with the organomercurial PCMS, and since the dose–response characteristics of the induction of efflux were almost identical to the inhibition of uptake by some of these analogs (Fig. 2), it was concluded that both efflux stimulation and uptake inhibition by these agents were the result of a direct interaction of the ACPC and ACHC derivatives with the active site of the transport carriers. This conclusion was also supported by the demonstration that the most potent inhibitor of GABA uptake, *trans*-3-ACPC, acted as a competitive inhibitor of the GABA transport process (Fig. 1).

The actions of some of the acyclic GABA analogs on the GABA uptake and efflux systems were very similar to those observed for the ACPC and ACHC derivatives. On the other hand, nipecotic acid, the very potent GABA uptake inhibitor, exhibited an apparent dissociation of its inhibition of GABA uptake from its stimulation of carrier-mediated GABA efflux (Fig. 2). The structural analog of nipecotic acid, 3-piperidyl acetic acid, however, produced effects very similar to those of *trans*-3-ACPC and 4-amino-3-hydroxybutanoic acid (Fig. 2). The strong inhibition of GABA uptake produced by nipecotic acid was considered to be a mixed type of inhibition that was due, in part to an interaction of this compound with membrane sites that impeded, rather than activated, the GABA carrier function. This explanation was based on the similarity of the effects of nipecotic acid to the actions of the local anesthetics tetracaine and quinacrine. These agents also produced strong inhibition of GABA uptake when they were present in high concentrations in the assay medium, but neither agent caused stimulation of [^{3}H]GABA efflux. These findings suggest that the local anesthetics were acting in a non-competitive manner that prevented any carrier movement across the membrane bilayer. Such an action would lead to a decrease in both GABA uptake and GABA efflux. This scheme was supported by the observation that tetracaine acted as a non-competitive inhibitor of GABA uptake (Fig. 1). Benzodiazepine inhibition of GABA uptake may also have been the result of similar non-competitive inhibition by these agents, or it may have been due to the activation by these compounds of membrane Cl^- channels [18]. It has been shown previously that GABA transport into synaptic membrane vesicles is dependent not only on the [Na^+] but also on the [Cl^-] gradient [12].

The order of the affinities for the interaction of the ACPC and ACHC analogs with the carrier site determined in the present study (*trans*-3-ACPC > *cis*-3-ACPC > *cis*-3-ACHC > *trans*-2-ACPC ≃ *cis*-2-ACPC ≫ *trans*-3-ACHC ≃ *cis*-4-ACHC ≃ *trans*-4-ACHC) was somewhat similar to the reported potencies of these amino acid analogs for activation of the physiologic GABA receptor sites in spinal neurons (*trans*-3-ACPC > *cis*-3-ACPC ≫ *cis*-3-ACHC ≫ *trans*-2-ACPC ≫ *cis*-2-ACPC ≃ *cis*-4-ACHC ≃ *trans*-4-ACHC) [19]. These relationships indicate that the 3-ACPC isomers are most effective in interacting with either uptake or receptor sites and that the *trans*-3-ACPC conformation is the most active isomer in both processes. It would appear then that the nearly extended conformation of GABA is the preferred conformation for initial binding to either the carrier or to the receptor active site. The distinction between extended versus folded conformations of GABA must be especially important for binding at the receptor sites since the activity of *trans*-3-ACPC at these sites was four to seven times greater than that of the *cis*-3-analog [19].

On the other hand, the difference between *trans*-3-ACPC and *cis*-3-ACPC inhibition of GABA uptake was modest (the *trans*-isomer was two to four times more potent than the *cis*-isomer). A clearer distinction between the characteristics of the uptake and receptor GABA recognition sites in synaptic membranes was obtained by examining the activity of some of the other cyclic analogs of GABA. Both *cis*- and *trans*-2-ACPC, as well as *cis*-3-ACHC, were relatively good inhibitors of the GABA transport carrier, whereas *cis*-2-ACPC is totally inactive, and *trans*-2-ACPC and *cis*-3-ACHC are very weak GABA receptor agonists [19]. The same agents also are weak or inactive inhibitors of the Na^+-independent (GABA receptor) binding activity of brain neurons [3].

The selectivity of the active site of the GABA uptake carrier for the extended conformation of the cyclic GABA analogs was greater than the selectivity for the acyclic GABA analogs. In terms of inhibition of GABA uptake, 3-guanidinopropanoic acid was equipotent to 4-amino-3-hydroxybutanoic and 5-aminopetanoic acid. Only 6-aminohexanoic acid was a very weak carrier-blocking agent (Table 2). These observations tend to support the idea that the acyclic GABA analogs are rather flexible molecules, which are able to assume the appropriate conformations at the active site of the GABA uptake carrier.

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