0026-895X/94/030550-08$03.00/0

MOLECULAR PHARMACOLOGY, 46:550–557, 1994.

Stable Expression of a Neuronal γ-Aminobutyric Acid Transporter, GAT-3, in Mammalian Cells Demonstrates Unique Pharmacological Properties and Ion Dependence

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Received January 14, 1994; Accepted June 12, 1994

SUMMARY

The transport proteins that mediate γ-aminobutyric acid (GABA) reuptake have been major targets for the development of agents to treat neurological diseases such as epilepsy, where augmentation of GABAergic function is indicated. The recent isolation of cDNAs for four distinct brain GABA carriers has provided an avenue for creating more specific and selective antagonists of GABA transport. An LLC-PK$_1$ cell line stably expressing GABA transporter type 3 (GAT-3), a β-alanine-sensitive neuronal GABA transporter, has been generated and used to examine the kinetics, ion dependence, and pharmacological properties of the transporter. In this cell line, the GAT-3 carrier transports GABA with an apparent K_m of 4 μM and a V_{max} of 1.25×10^{-16} mol/cell/min. β-Alanine is a relatively potent inhibitor of GAT-3 GABA transport, with a K_i value of 34 μM. β-Alanine also serves as a substrate for the carrier (K_m = 29 μM, $V_{max} = 1.82 \times 10^{-16}$ mol/cell/min) and appears to interact with the transporter at the same or a similar site as GABA. Other experimental GABA transport antagonists developed as anticonvulsant agents, including tiagabine, CI-966, SKF-100330-A, SKF-89976-A, and NO-711, are weak inhibitors of GAT-3 GABA transport, suggesting that their therapeutic effects may be more related to their ability to block GABA transporters other than GAT-3. GAT-3 exhibits a sigmoidal dependence on Na^+ concentration, with a Hill coefficient of 1.65, suggesting that more than one Na^+ ion is involved in the transport mechanism. In contrast, the transport activity shows a hyperbolic Cl^- dependence, with a Hill coefficient of 1.05. The K_m for Cl^- is 78 mM, a value severalfold higher than has been noted for another cloned GABA carrier, GABA transporter type 1. Interestingly, for GAT-3 a reduction of the Cl^- concentration results in a small but consistent increase in the apparent K_m for GABA, suggesting that the interaction of chloride with the transporter may be an important initial event in the mechanism of transport. These results underscore the unique properties of GAT-3 and distinguish this transporter as a new target for the development of GABA-mimetic agents.

GABA, the major inhibitory neurotransmitter in the mammalian brain, is efficiently cleared from synapses and the extracellular space by high affinity transporters present on neurons and glial cells. The processes responsible for GABA reuptake have been studied in detail in the past in a variety of preparations, including synaptosomes, brain slices, and primary cultures of cells (1–4). At least three GABA transporter cDNAs have been isolated from rat brain, i.e., GAT-1 (an ACHC-sensitive GABA transporter, also referred to as GAT-A) (5), GAT-3 (a β-alanine-sensitive GABA transporter, also referred to as GAT-B) (6, 7), and GAT-2 (7). A fourth transporter, BGT-1, isolated from canine kidney (8) also transports GABA but with a much lower apparent affinity than brain GABA uptake systems. Four GABA transporter cDNAs have been isolated from murine brain and are termed mGAT-1 through mGAT-4 (9, 10). mGAT-1, -3, and -4 are the murine homologues of GAT-1, -2, and -3, respectively. mGAT-2 is most similar in sequence to BGT-1 but appears to be found in mouse

This work was supported in part by a Hitchings Award from the Burroughs Wellcome Fund and National Institutes of Health Grant DA07595. J.A.C. is the recipient of an Advanced Predoctoral Fellowship from the Pharmaceutical Manufacturers' Association Foundation.

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ABBREVIATIONS: GABA, γ-aminobutyric acid; GAT-1–3, γ-aminobutyric acid transporter types 1–3; mGAT-1–4, murine γ-aminobutyric acid transporter types 1–4; ACHC, *cis*-1,3-aminocyclohexanecarboxylic acid; L-DABA, L-diaminobutyric acid; KRH, Krebs-Ringer-HEPES; DMSO, dimethylsulfoxide; CI-966, 1-[2-[bis[4-(trifluoromethyl)phenyl]methoxy]ethyl]-1,2,5,6-tetrahydro-3-pyridinecarboxylic acid; THPO, 4,5,6,7-tetrahydroisoxazolo[4,5-*c*]pyridin-3-ol; NO-711, 1-[2-[[(diphenylmethylene)amino]oxy]ethyl]-1,2,5,6-tetrahydro-3-pyridinecarboxylic acid; tiagabine, (*R*)-*N*-[4,4-bis(3-methyl-2-thienyl)but-3-en-1-yl]nipecotic acid; SKF-89976-A, 1-(4,4-diphenyl-3-butenyl)-3-piperidinecarboxylic acid; SKF-100330-A, 4-(3-carboxy-1,2,5,6-tetrahydropyridino)-1,1-diphenyl-1-butene; SKF-100561, 1-(4,4-diphenyl-3-butenyl)-3-pyrrolidineacetic acid; β-proline, 3-pyrrolidinecarboxylic acid; α-MEM, Eagle minimum essential medium, α modification; BGT-1, betaine transporter type 1; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

brain as well as kidney and other peripheral tissues. GAT-1, GAT-2, and GAT-3 can be distinguished based on their pharmacological profiles (6, 7). Furthermore, differences in the expression of GAT-1 and GAT-3 mRNA in brain have been shown (6, 11). High levels of GAT-1 mRNA are found in all areas of the cerebral cortex and the hippocampal formation (11), areas where levels of GAT-3 mRNA expression are low (6). Transport mediated by the GAT-1 clone exhibits many of the properties of brain GABA transport described in the literature (2, 12, 13), including L-DABA and ACHC sensitivities. However, the properties of GAT-3 do not resemble any previously described GABA transport mechanism.

Disruption of GABAergic neurotransmission has been linked to several neurological and psychiatric disorders, including epilepsy (14–16), schizophrenia (17–19), and Huntington's disease (20). It has been suggested that agents known to enhance GABAergic transmission may serve as useful antinociceptives (21). Many anticonvulsants used for the treatment of epilepsy are designed as GABA-mimetics, acting as GABA receptor agonists, as inhibitors of GABA metabolism, or as inhibitors of GABA reuptake (22–24). Although many GABA transport inhibitors have been shown to have anticonvulsant properties (25), the particular transporter subtypes affected by these agents remain to be determined.

Several studies have examined in detail the stoichiometry and ion dependence of GABA transport in synaptosomes and brain slices (1, 4, 26), but these issues have only recently begun to be addressed using transporter cDNAs heterologously expressed in various expression systems (27–29). In particular, studies with GAT-1 expressed in *Xenopus* oocytes (28, 29) have confirmed earlier predictions (for review, see Ref. 26) that transport of a single GABA molecule is accompanied by the cotransport of two sodium ions and one chloride ion. The stoichiometry and ion dependence of GABA transport by GAT-2, GAT-3, BGT-1, or the murine transporter homologues have yet to be reported.

In a previous report it was shown that GAT-3 expressed in HeLa cells exhibited a high affinity transport process that was sensitive to β-alanine and was found predominantly in neurons (6). In an effort to further establish the differences between the properties of GAT-3 and GAT-1 transport, we have stably expressed GAT-3 in LLC-PK$_1$ cells and examined ion dependence, substrate selectivity, and sensitivity to anticonvulsant agents.

Experimental Procedures

Plasmid construction. GAT-3 was excised from pGAT3 (6) by restriction digestion with *Xho*I. The excised GAT-3 was ligated into *Xho*I-prepared JG3.6 (30), resulting in the formation of the plasmid pGAT3JG.

Stable expression of GAT-3. LLC-PK$_1$ cells, a porcine kidney cell line, were cultured in α-MEM (JRH Biosciences), supplemented with 10% fetal bovine serum (Hyclone) and 1% penicillin/streptomycin (GIBCO/BRL), at 37° in 5% CO_2. LLC-PK$_1$ cells grown to 50% confluency in 10-cm tissue culture plates were used for control and GAT-3 transfections. LLC-PK$_1$ cells were exposed to either a solution of cationic lipid/DNA (Lipofectin; GIBCO/BRL) in OPTI-MEM I medium (GIBCO/BRL) (20 μg of Lipofectin/9 μg of pGAT3JG/1 μg of pSVNEO; 2:1 Lipofectin to DNA ratio) or a solution containing cationic lipid alone (20 μg of Lipofectin) in OPTI-MEM I medium, for 8 hr at 37°. After incubation, OPTI-MEM I solutions were removed and replaced with serum- and penicillin/streptomycin-supplemented α-MEM. Cells were allowed to recover for several hours, after which each 10-cm plate of cells was split to five 10-cm plates. After an overnight incubation, cells were exposed to 500 μg/ml geneticin (G418; GIBCO/BRL) for a period of 2 weeks. Resistant colonies were isolated using sterile cotton swabs (31) and transferred to 24-well plates. Those cells that survived the isolation procedure were cultured for transport assays to screen for expression of GAT-3. After the identification of cells expressing GAT-3, these cells were grown in serum- and penicillin/streptomycin-supplemented α-MEM containing a maintenance dose of G418 (200 μg/ml).

Transport assay. Cells were washed with a modified KRH buffer containing either sodium chloride or choline chloride (120 mM NaCl or choline chloride, 4.7 mM KCl, 2.2 mM $CaCl_2$, 10 mM HEPES, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 5 mM Tris, pH adjusted to 7.4 with 1 M KOH) (32). A KRH buffer containing no chloride was made by isotonic substitution of sodium acetate for NaCl, potassium acetate for KCl, and calcium acetate for $CaCl_2$. Concentrations of sodium and chloride were varied in buffers for the saturation studies by diluting NaCl-containing KRH buffer into buffers that lacked sodium and chloride, respectively. Cells were preincubated in 0.5 ml (24-well plates) of the indicated KRH medium for 15 min at 37°. A 10× cocktail of [^{3}H]GABA (20–50 nM final concentration; Amersham or New England Nuclear) or β-[^{3}H]alanine (25–50 nM final concentration; New England Nuclear), with or without unlabeled competitors, was added and cells were incubated for 20 min at 37°, unless otherwise noted. Transport was terminated on ice by two 1-ml washes with the specified ice-cold modified KRH buffer. Cells were lysed with 0.5 ml of 1% sodium dodecyl sulfate and the lysate radioactivity was measured by liquid scintillation counting. Average uptake from replicate experiments was determined and expressed as moles/cell/minute, except where indicated. Background levels of [^{3}H]GABA or β-[^{3}H]alanine uptake were determined from uptake assays using nontransfected LLC-PK$_1$ cells, and these values were subtracted from those of the transfected cells to obtain net [^{3}H]GABA or net β-[^{3}H]alanine uptake. All drugs in these studies were dissolved in the appropriate assay buffer with the exception of CI-966, which was dissolved at 1 mg/ml in DMSO and then diluted into the appropriate assay buffer for the desired concentrations. When DMSO was used as solvent, controls were performed with the appropriate concentration of DMSO with no drug.

Results

GAT-3 transport kinetics in LLC-PK$_1$ cells. The GAT-3 cDNA was subcloned into the vector JG3.6 (30) under the direction of the cytomegalovirus promoter, to yield the plasmid GAT3JG. pGAT3JG was used to transfect a porcine kidney cell line, LLC-PK$_1$, to generate a mammalian cell line stably expressing GAT-3. The transport of [^{3}H]GABA by LLC-PK$_1$ cells transfected with pGAT3JG was 150–200-fold above that determined for the parental cell line (data not shown), indicating that transport was due to expression of the GAT-3 cDNA. Transport of GABA was time dependent, increasing linearly to 90 min, and required the presence of both Na^+ and Cl^- ions. GAT-3 GABA transport was saturable, with an apparent K_m for transport of 4.1 ± 0.55 μM and a V_{max} of 1.25 ± 0.14 × 10^{-16} mol/cell/min (Fig. 1A). GABA saturation analyses in the presence and absence of a fixed concentration of β-alanine (50 μM), a relatively potent inhibitor of GAT-3 GABA transport, were performed to determine whether GABA and β-alanine interact at the same or similar site(s) on the carrier protein. The results show that β-alanine had little effect on the maximum velocity of GABA transport but decreased the apparent affinity of the transporter for GABA by doubling the K_m value (Fig. 1B). These findings suggest that GABA and β-alanine compete for the same or similar site(s) on GAT-3.

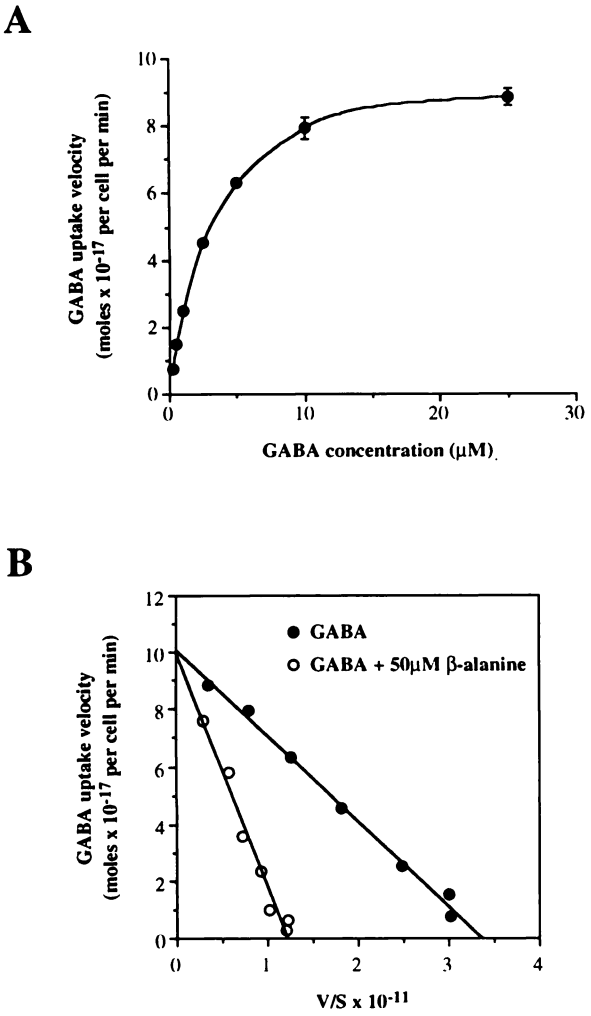

Fig. 1. Saturation analyses of [^{3}H]GABA transport in LLC-PK$_1$ cells expressing pGAT3JG. A, Saturation analysis of [^{3}H]GABA transport; B, Eadie-Hofstee transformation of saturation data for [^{3}H]GABA transport, with or without 50 μM β-alanine. GABA saturation data are representative of those obtained in four separate experiments; GABA saturation data obtained in the presence of β-alanine are representative of data from two separate experiments. Each *point* represents the mean of triplicate determinations, with a standard error of ≤10%. K_i and V_{max} values for GABA transport in the absence of β-alanine are the mean ± standard error of four separate determinations, and values obtained in the presence of β-alanine are the mean ± standard error of two separate determinations.

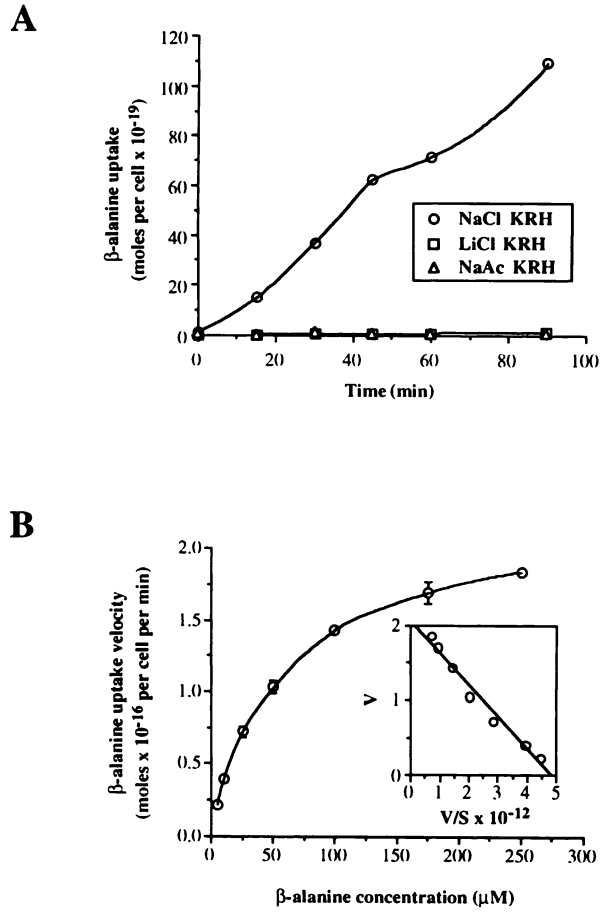

Fig. 2. Characterization of β-[^{3}H]alanine transport in LLC-PK$_1$ cells expressing pGAT3JG. A, Time course and Na^+ and Cl^- dependence of β-[^{3}H]alanine transport. *NaCl KRH*, modified KRH buffer; *LiCl KRH*, modified KRH buffer with isotonic replacement of NaCl by LiCl; *NaAc KRH*, modified KRH buffer with isotonic replacement of sodium acetate for NaCl, potassium acetate for KCl, and calcium acetate for $CaCl_2$. Each *point* is the mean of triplicate determinations, with a standard error of ≤10%. B, Saturation analyses of β-[^{3}H]alanine transport. *Inset*, Eadie-Hofstee transformation of saturation data. β-Alanine saturation data are representative of those obtained in three separate experiments; each *point* represents the mean of triplicate determinations, with a standard error of ≤10%. K_m and V_{max} values are the mean ± standard error of three separate determinations.

LLC-PK$_1$ cells transfected with pGAT3JG not only transport GABA but also demonstrate a consistent 5–6-fold increase in the ability to transport β-[^{3}H]alanine, compared with the parental cell line (data not shown). Transport of β-alanine continued to increase with time to maximal incubations of 90 min (Fig. 2A) and was dependent on the presence of both Na^+ and Cl^- ions. GAT-3 β-alanine transport was saturable, exhibiting an apparent K_m for transport of 29.3 ± 7.2 μM and a V_{max} of 1.82 ± 0.3 × 10^{-16} mol/cell/min (Fig. 2B). A background β-alanine transport process was detected in LLC-PK$_1$ cells and is distinguished from GAT-3 β-alanine transport by its higher apparent K_m value (64 ± 9.3 μM) and its sensitivity to taurine (K_i < 50 μM) (data not shown).

Pharmacological characterization of GAT-3 transport. Previous studies have shown that GAT-3 GABA transport is pharmacologically distinct from other GABA transport processes (6, 7). In agreement with these previous reports, β-alanine was found to be a relatively strong inhibitor of GAT-3 GABA transport in LLC-PK$_1$ cells, exhibiting a K_i value of 43 ± 9 μM (Table 1), and ACHC was a weak inhibitor, with a K_i value of >1 mM (Table 1). β-Proline, a GABA uptake inhibitor that has been shown to have slightly greater selectivity for glial than neuronal GABA transport processes (22, 34), inhibited GAT-3 GABA transport with a K_i value of 194 μM. A more lipophilic derivative of homo-β-proline, SKF-100561 (35), was a relatively weak inhibitor, exhibiting a K_i value of 331 μM. Additional agents that interact with GABA transporters, such as arecaidine (36), betaine (8), and valproic acid (37), were all weak inhibitors of GAT-3 GABA transport, with K_i values of >1 mM (Table 1). The $GABA_A$ receptor agonist muscimol was similarly unable to significantly affect GAT-3 GABA transport at concentrations up to 5 mM. Finally, the amino acids DL-alanine and DL-proline had no effect on GABA transport in LLC-PK$_1$ cells (Table 1).

Several GABA transport inhibitors have been shown to have

TABLE 1

Inhibitor sensitivity of GABA and β-alanine uptake in LLC-PK$_1$ cells expressing pGAT3JG

K_i values are presented as means ± standard errors and represent the average of at least three separate experiments. Values were calculated from IC_{50} determinations according to the method of Cheng and Prusoff (33).

Inhibitor	K_i	
	GABA uptake	β-Alanine uptake
	μM	
GABA	ND[a]	3 ± 1
β-Alanine	43 ± 9	ND
β-Proline	201 ± 25	ND
L-DABA	ND	207 ± 83
SKF-100561	331 ± 40	ND
Arecaidine	1590 ± 120	ND
ACHC	>1000	2270 ± 610
Taurine	ND	2630 ± 830
Betaine	3800 ± 1004	ND
Valproic acid	>5000	ND
Muscimol	>5000	ND
DL-Alanine	>5000	ND
DL-Proline	>5000	ND

[a] ND, not determined.

anticonvulsant properties in animal seizure models (25, 35, 38–41). Although most of these agents have been shown to inhibit GABA transport with nanomolar potencies in rat synaptosomal preparations, brain slices, and primary cultures of neurons and glia (25, 38, 39, 42, 43), the identity of the transporter(s) with which these agents interact remains unclear. The inhibition of GAT-3 GABA transport by six anticonvulsant agents was examined in LLC-PK$_1$ cells. THPO, a relatively weak inhibitor of GABA transport, was found to be equipotent at inhibiting GABA transport by GAT-1 and GAT-3 in previous studies (6). In the current studies GAT-3 GABA transport was also weakly inhibited by THPO, with a K_i value of 664 μM (Table 2). SKF-100330-A (35), a lipophilic derivative of guvacine, exhibited a K_i value of 191 μM. SKF-100330-A was 10-fold less potent at inhibiting GAT-3 GABA uptake than was guvacine (6). Previous studies showed that NO-711, a nipecotic acid derivative (38), exhibited nanomolar potency for inhibiting GAT-1 GABA transport, whereas GAT-3 transport was 5 orders of magnitude less sensitive to this agent (6). Similarly, in GAT-3-expressing LLC-PK$_1$ cells, NO-711 was a weak inhibitor of GABA transport, with a K_i value of >900 μM (Table 2). In addition, the moderately lipophilic nipecotic acid derivatives tiagabine (NO-328) (40, 42) and SKF-89976-A (35) were weak inhibitors of GAT-3 GABA transport (Table 2). Because of the limited solubility of CI-966, a guvacine derivative, an IC_{50} value was unattainable. However, it was possible to determine that the K_i of CI-966 was >10 μM, a concentration well above those in the nanomolar range reported for inhibition of GABA transport in several assay systems (Table 2). These findings suggest that those anticonvulsants examined here that are potent inhibitors of GABA transport exert their pharmacological effects on GABA transporter subtypes other than GAT-3.

Examination of the sensitivity of GAT-3 β-alanine transport to select agents revealed that this process was potently inhibited by GABA, with a K_i value of 3 μM (Table 1). L-DABA was a much less potent inhibitor of β-alanine transport in LLC-PK$_1$ cells (K_i = 207 μM) (Table 1). β-Alanine transport was virtually insensitive to ACHC (K_i = 2 mM) (Table 1). Finally, taurine was found to be a very weak inhibitor of β-alanine transport, with a K_i value of 3 mM (Table 1). Poor inhibition by taurine of GAT-3 β-alanine transport distinguishes this transport process from that of a recently isolated cDNA that encodes a transporter capable of utilizing both β-alanine and taurine as substrates (44, 45).

Ion dependence of GABA transport. The dependence of GAT-3 GABA transport on Na^+ ions resembles that described for GAT-1 GABA transport in several previous reports (27–29). GABA transport by GAT-3 increased as a sigmoidal function of the Na^+ concentration, with a Hill coefficient of 1.65 ± 0.56 and an apparent K_m of 69 ± 32 mM (Fig. 3A). In recent studies of GAT-1 GABA-induced currents in *Xenopus* oocytes, transport showed a sigmoidal dependence on Na^+, with a Hill coefficient of 1.74 and an apparent K_m of 73 mM (28). In the present work, decreasing Na^+ to millimolar concentrations significantly reduced GABA transport, and isotonic replacement of NaCl with LiCl in the assay buffer abolished uptake. GABA saturation analyses performed with a half-maximal concentration of Na^+ (70 mM) resulted in reduction of the maximal velocity for transport to approximately half ($1.32 \pm 0.21 \times 10^{-16}$ mol/cell/min) that observed in the presence of a normal Na^+ concentration (120 mM) ($2.23 \pm 0.44 \times 10^{-16}$ mol/cell/min) (Table 3). In contrast, this lower Na^+ concentration (70 mM) had no effect on the K_m value for GABA transport (Table 3).

TABLE 2

Comparison of inhibitor sensitivity of GABA uptake by LLC-PK$_1$ cells expressing pGAT3JG with reported values obtained in various assay systems

K_i values for LLC-PK$_1$ cells expressing pGAT3JG are the average ± standard deviation of values calculated, according to the method of Cheng and Prusoff (33), from the IC_{50} values determined in at least two experiments.

	K_i, GAT-3	IC_{50}			
		Brain slices	Synaptosomes	Cultured neurons	Cultured astrocytes
	μM		μM		
SKF-100330-A	191 ± 14	ND[a]	0.21[b]	ND	ND
Tiagabine	362 ± 27	ND	0.067[c]	0.446[c]	0.182[c]
SKF-89976-A	203 ± 20	ND	0.20[b]	ND	ND
THPO	664 ± 49	5000[d]	ND	ND	300[d]
CI-966	>10	ND	0.104[e]	4.64[e]	0.304[e]
NO-711	>900	ND	0.047[f]	1.238[f]	0.636[f]

[a] ND, not determined.
[b] From Ref. 35.
[c] From Ref. 42.
[d] From Ref. 25.
[e] From Ref. 39.
[f] From Ref. 38.

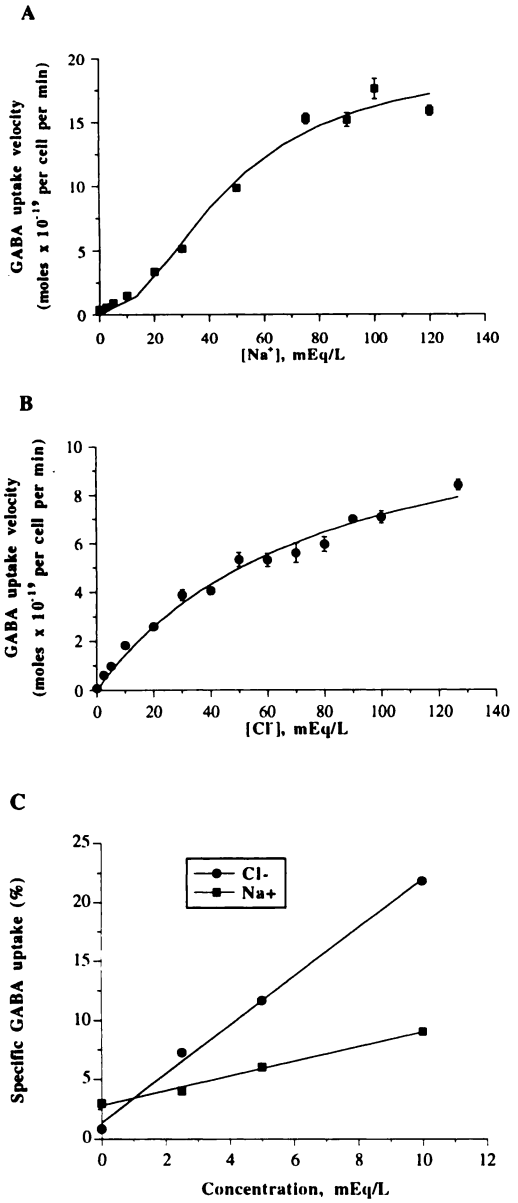

Fig. 3. Dependence of [^{3}H]GABA transport on Na^+ and Cl^- concentrations. A and B, Sodium (A) and chloride (B) concentration dependence of [^{3}H]GABA transport. GABA uptake velocity is plotted against the Na^+ concentration present in the assay buffer. Na^+ concentrations were varied by mixing LiCl-containing KRH buffer with NaCl-containing KRH buffer, and Cl^- concentrations were varied by mixing sodium acetate-containing KRH buffer with NaCl-containing KRH buffer, as described in Experimental Procedures. C, GABA uptake velocity at ion concentrations of 0–10 mM, plotted as a percentage of control GABA uptake (120 mM Na^+, 126.9 mM Cl^-). Data are representative of those obtained in three separate experiments; each *point* represents the mean of triplicate determinations, with a standard error of ≤10%.

Because reduction of the Na^+ concentration had little effect on the K_m for GABA transport, interaction of Na^+ ions with the transporter must have no effect on substrate binding but instead may be involved in shuttling the loaded carrier to the intracellular environment. These data indicate that GAT-3 GABA transport in LLC-PK_1 cells has a strict dependence on Na^+ ions; furthermore, they suggest that more than one Na^+ ion may be cotransported in each cycle.

The Cl^- dependence of transport by GAT-3 showed a hyperbolic relationship, with a Hill coefficient of 1.05 ± 0.03 and an apparent K_m of 78 ± 2.5 mM (Fig. 3B). Work in *Xenopus* oocytes has shown that GAT-1 GABA transport-induced currents exhibit a hyperbolic relationship with respect to Cl^- dependence, with a Hill coefficient of 0.71 (28). In contrast, the apparent K_m for GAT-1 Cl^- transport was 19 mM (28). Decreasing the Cl^- concentration of the assay buffer to 0–10 mM reduced GAT-3 GABA uptake to a much lesser extent than did reduction of the Na^+ concentration (Fig. 3C). This result is of interest because GABA transport by GAT-3 expressed in HeLa cells was not detectable at Cl^- concentrations of 6.9 mM or lower (6). In the current study, only complete removal of Cl^- from the assay buffer abolished transport. Saturation studies of GABA transport in the presence of a half-maximal concentration of Cl^- (80 mM) revealed a small but consistent increase in the apparent K_m value but little effect on the V_{max} value (Table 3). These data suggest that a single Cl^- ion is necessary for the transport of a single GABA molecule and that Cl^- ions have an effect on the interaction of GABA with the transport molecule.

TABLE 3

Effects of half-maximal Na^+ and Cl^- concentrations on apparent K_m and V_{max} values for GAT-3 GABA transport

K_m and V_{max} values are the mean ± standard error of five separate experiments for assays done in 120 mM Na^+/127 mM Cl^- and 70 mM Na^+/127 mM Cl^- and four separate experiments for assays done in 120 mM Na^+/80 mM Cl^-.

KRH Buffer Ion Concentrations	K_m	V_{max}
	μM	mol × 10^{-16}/cell/min
120 mM Na^+/127 mM Cl^-	5.12 ± 0.59	2.24 ± 0.44
70 mM Na^+/127 mM Cl^-	5.12 ± 0.67	1.32 ± 0.21
120 mM Na^+/80 mM Cl^-	7.39 ± 0.69	2.33 ± 0.69

Discussion

The GAT-3 GABA transporter is one of several carrier proteins in the mammalian central nervous system (5–7, 9, 10) that are responsible for the rapid removal of GABA from synaptic and extracellular spaces. These high affinity transporters, found on both neurons and glia, are thought to play a significant role in the termination of GABAergic signaling. Previous reports have shown GAT-3 to be a gene product structurally and functionally distinct from two other cloned carriers, GAT-1 and GAT-2 (6, 7). Preliminary studies have demonstrated that GABA transport by all three of these proteins is dependent on both Na^+ and Cl^- ions, and the three transporters exhibit relatively low apparent K_m values for GABA; however, their pharmacological profiles, substrate specificity, and degree of Cl^- dependence differ. For example, GAT-1 is characterized by its sensitivity to ACHC, whereas GAT-3 is most sensitive to β-alanine. In addition, although the distribution of GABA transporter mRNAs in the brain has been shown to be heterogeneous, there are particular regions where one GABA transporter mRNA may be present and another absent (6, 11). For example, a recent study has demonstrated significant expression of GAT-1 mRNA in the cerebellum (11), whereas cerebellar GAT-3 mRNA is restricted to the deep cerebellar nuclei (6). To further examine the distinguishing characteristics of transport by GAT-3, a mammalian cell line stably expressing the plasmid pGAT3JG was established.

In addition to transporting [^{3}H]GABA with high affinity, LLC-PK_1 cells expressing pGAT3JG demonstrated a β-alanine transport activity that was dependent on both sodium and

chloride. Although previous work has shown that GAT-3 GABA transport expressed in HeLa cells is most potently inhibited by β-alanine (6), β-[^{3}H]alanine uptake was not detected above background HeLa cell levels. The presence of significant background β-alanine uptake by nontransfected HeLa cells (2.59×10^{-19} mol/cell/min) may have obscured expressed GAT-3 β-alanine transport. The increase in intracellular levels of β-alanine due to both endogenous and exogenous transporter uptake may have had a significant impact on the net influx of β-alanine. Although efflux of GABA through GAT-3 has yet to be reported, reversal of GABA transport processes does occur and has been studied extensively (for review, see Ref. 26). Alternatively, inherent differences in the manner in which HeLa cells and LLC-PK$_1$ cells express GAT-3 and associated proteins may also contribute to the apparent variation in substrate specificity between these two cell lines.

GAT-3 β-alanine transport in LLC-PK$_1$ cells exhibited a relatively low apparent K_m value and was potently inhibited by GABA. The GABA transport inhibitor ACHC was a poor inhibitor of β-alanine uptake, and L-DABA showed a moderate ability to inhibit this uptake process. Finally, taurine was also a weak inhibitor of GAT-3 β-alanine transport. These data distinguish GAT-3 β-alanine transport from the β-alanine uptake found in primary cultures of astrocytes (46). Although GABA was shown to be a competitive inhibitor of β-alanine transport in primary cultures of astrocytes, the apparent K_m for transport was higher (68 μM) (46) and IC_{50} values for GABA and L-DABA were approximately 1 mM (46). Taurine, also a substrate for the astrocytic β-alanine transport activity, exhibited an IC_{50} value of <1 mM (46). Therefore, GAT-3 is unlikely to be responsible for the β-alanine transport activity observed in primary cultures of astrocytes, a process that has properties most consistent with those of the recently cloned taurine transporter (44, 45). A previous study (3) reporting glial β-alanine transport in synaptosomes from whole rat brain, cortical slices, and sensory ganglia revealed an uptake process with kinetic and pharmacological characteristics similar to those described here for GAT-3. *In situ* hybridization analyses of GAT-3 mRNA distribution in the brain indicated that GAT-3 was expressed predominantly in neurons (6), although low GAT-3 mRNA expression in glia has not been completely ruled out. Immunohistochemical analyses will be informative in determining whether novel glial GABA transporters exist. Furthermore, given that this carrier transports both GABA and β-alanine, additional endogenous substrates for GAT-3 may be identified. However, the apparent high affinity exhibited by GAT-3 for GABA, the strong primary sequence homology between GAT-3 and other GABA transporters, and the localization of GAT-3 mRNA to select populations of GABAergic cell bodies argue that GABA serves as a physiological substrate for this transporter.

The kinetic properties of the uptake of GABA by LLC-PK$_1$ cells expressing pGAT3JG were similar to those previously reported for GAT-3 expressed in HeLa cells using the vaccinia/T7 RNA polymerase system (6). The current work shows that GAT-3 GABA transport is potently inhibited by β-alanine but is only weakly affected by the presence of ACHC. Addition of a fixed concentration of β-alanine to GABA saturation analyses revealed that GABA and β-alanine compete for the same or similar sites on the transport protein. The compounds initially tested for their ability to inhibit GAT-3 transport in HeLa cells demonstrate the same rank order of potency for inhibition of GABA uptake in LLC-PK$_1$ cells (6). Several additional agents were tested for their ability to interfere with GAT-3 GABA transport. Betaine, an osmolyte in the kidney that is transported by a carrier that also transports GABA (8), was a poor inhibitor of GAT-3 GABA transport. Arecaidine, like guvacine, is a constituent of the *Areca* nut, and both have been shown to inhibit GABA uptake in mini-slice preparations with submillimolar potencies (36). In the present study GAT-3 GABA transport was weakly affected by arecaidine, with a K_i value in the millimolar range.

β-Alanine, a substrate for GAT-3 and the most potent inhibitor of GAT-3 GABA transport, has long been thought of as a marker for glial GABA carriers (3). In addition to β-alanine, it has been suggested that β-proline is a specific inhibitor of glial GABA transport (34). The potency of β-proline inhibition of GABA transport ranges from an IC_{50} of 320 μM in cultured astrocytes from mouse brain (34) to an IC_{50} of 1.2 mM in mouse cerebral cortical mini-slices, which are thought to evince primarily neuronal uptake (47). β-Proline was more potent at inhibiting neuronal GAT-3 GABA transport in the current study (K_i = 194 μM) than at inhibiting GABA transport in cultured astrocytes (34). These data further demonstrate a complexity in GABA transport processes that was unexpected before the identification of cDNAs for these carriers. Pharmacological characterization of heterologously expressed GABA transporters and determination of mRNA tissue distributions (5–10) have revealed that carrier classification as neuronal or glial is too simplistic to describe the diversity of carriers that exist. Indeed, *in situ* hybridization studies have now shown that GAT-1 mRNA, originally thought to encode a neuronal GABA transporter based on its pharmacology (5), is expressed in a discrete population of glia as well as neurons (11).

A variety of compounds capable of altering GABA function were tested for their ability to inhibit GAT-3 GABA transport. The 4,4-diphenyl-3-butenyl derivative of homo-β-proline, SKF-100561, has been shown to have submicromolar potency for inhibition of synaptosomal GABA transport (25). SKF-100561 had only moderate potency at inhibiting GAT-3 GABA transport, with a submillimolar K_i value. Finally, the compounds valproic acid and muscimol, agents known to interact with GABA transport and receptor function (37), had no effect on GAT-3 GABA transport. Similarly, the amino acids DL-alanine and DL-proline did not affect GABA uptake in these studies.

Alterations in GABA neurotransmission have been linked to epilepsy (15, 48). Certain GABA uptake inhibitors have been shown to possess anticonvulsant properties (25) (38–41), presumably by augmenting GABAergic neurotransmission. Although these agents have been shown to have potent effects on GABA transport in several assay systems (25, 38, 39, 42), the identity of the transporter(s) with which these agents interact remains to be determined. Previous work demonstrated that THPO, a relatively weak GABA uptake inhibitor, had equipotent effects on GABA transport by GAT-1 or GAT-3 (6), and these data correspond well to the IC_{50} value for inhibition of GABA transport in cultured astrocytes (25). Therefore, it is possible that the pharmacological effects of THPO result from interaction with GAT-1 and/or GAT-3 transport proteins. In contrast, the agents NO-711, CI-966, tiagabine, SKF-89976-A, and SKF-100330-A have been shown to have nanomolar to submicromolar potencies at inhibiting GABA uptake in several

assay systems (25, 35, 38, 39, 42, 43). Data presented here indicate that these agents are not potent inhibitors of GAT-3 transport and most likely produce their anticonvulsant effects by interacting with GAT-1, GAT-2, or as yet unidentified GABA transporters.

The ion dependence of GABA transport processes studied by the use of preparations such as syntaptosomes and brain slices is well documented and provides insight into the mechanistic features of transport (for review, see Ref. 26). It is well established that the transport of Na^+ down a concentration gradient drives the uphill transport of GABA across the cell membrane. Transport of GABA requires the cotransport of Cl^- in addition to Na^+, (1, 4, 49). Studies of GAT-1 exogenously expressed in mammalian cells (27) and *Xenopus* oocytes (28, 29) have shown that the Na^+ and Cl^- dependence of this transport process strongly resembles the Na^+ and Cl^- dependence of GABA transport previously described for synaptosomes, brain slices, and a partially purified carrier (1, 4, 49). To date, there have been no detailed reports on the stoichiometry and ion dependence of GAT-3 or GAT-2 GABA transport. The current study examines the ion dependence of GAT-3 GABA transport expressed in LLC-PK_1 cells. The change in transport as a function of varying Na^+ concentrations is reflected in a Hill coefficient of 1.65, consistent with the involvement of more than one Na^+ ion in the transport process. With an apparent K_m value of 69 mM for Na^+, GAT-3 exhibited a strict dependence on Na^+ that resembled that observed for GAT-1 in a previous study with *Xenopus* oocytes, which exhibited an apparent K_m of 73 mM (28). In contrast, the Cl^- dependence data revealed a Hill coefficient of 1.05, consistent with a single Cl^- ion requirement for GAT-3 GABA transport. In addition, the apparent K_m value for GAT-3 Cl^- transport observed in this study was significantly different from that observed for GAT-1 in a previous study with *Xenopus* oocytes (28) (78 mM and 19 mM, respectively). Data from a recent study in which removal of Cl^- from the assay buffer had different effects on GABA transport by GAT-1, GAT-2, and GAT-3 (7) support the current findings that GABA transporters may be distinguished on the basis of Cl^- dependence. Electrophysiological studies of GAT-1 transport in *Xenopus* oocytes have demonstrated an electrogenic process, with one molecule of GABA being cotransported with two Na^+ ions and one Cl^- ion in one transport cycle across the cell membrane (28, 29). Data from the preliminary studies of GAT-3 ion stoichiometry presented here suggest that this may also be an electrogenic process. Further examination of the differences in Cl^- dependence for each of the transporters may lead to a better understanding of the differences in transport function between GABA carriers.

A model for the cycle of GABA translocation across the cell membrane has been proposed based on analyses of uptake, efflux, and exchange studies with synaptic membrane vesicles (50) (for review, see Ref. 26). The model predicts that Cl^- first interacts with the carrier, resulting in a conformational change in the transport protein that, in turn, makes interaction of transporter and GABA favorable. After substrate binding, Na^+ ions interact with the carrier and the loaded carrier translocates ions and substrate to the intracellular compartment. Data obtained in the present studies examining the effects of half-maximal concentrations of Na^+ and Cl^- ions on the kinetics of GAT-3 GABA transport are consistent with such a model for uptake. Reduction of extracellular Cl^- concentrations resulted in a small but consistent increase in the apparent K_m for transport, as if interaction of GABA with the transporter were impaired. This may suggest that the Cl^- binding site is closely associated with the substrate binding site on the carrier. Alternatively, the transporter conformation may not be optimal for substrate binding under conditions of reduced extracellular Cl^- concentrations and therefore higher concentrations of GABA are required to drive the equilibrium towards net uptake. In contrast, reduction of the Na^+ concentration had no effect on the apparent K_m for GAT-3 GABA transport but resulted in a decrease in the maximal velocity for uptake. These results are consistent with the idea that a transporter loaded with Cl^- and substrate is not capable of completing the transport cycle in the absence of appropriate Na^+ concentrations. Additional kinetic and thermodynamic analyses of GAT-3 GABA transport will provide a more accurate estimate of the stoichiometry and binding order of substrates in the transport cycle.

The stable expression of GAT-3 in mammalian cells has provided a system for more complete examination of transport characteristics, ion dependence, and anticonvulsant sensitivity, for comparison with similar properties of other recently cloned GABA transporters. Studies such as these may help to elucidate the roles of individual GABA transporters in normal nervous system functioning. The physiological relevance of the heterogeneity of GABA transporters is not clearly understood, but such diversity of membrane proteins is not uncommon. Heterogeneity is common in receptor families, where molecular identification of receptor subtypes has often preceded determination of physiological significance. Immunohistochemical analyses at the cellular level will help to determine in which cell types these transporters are expressed and where in the cells transporters are targeted. Data from such experiments will contribute to understanding the context in which particular transporters are expressed and in turn may shed some light on the significance of heterogeneous forms of GABA transporters in normal brain function. Perhaps the development of drugs with specificity for particular GABA transport proteins will prove beneficial for the treatment of neurological disorders where augmentation of GABAergic function is indicated.

Acknowledgments

The authors would like to thank M. Lerner for his support and encouragement and W. A. Clark for his careful review of the manuscript. ACHC was generously provided by Research Biochemicals Inc., as part of the Chemical Synthesis Program of the National Institute of Mental Health [Contract 278–90–0007 (BS)]. CI-966 was the generous gift of C. P. Taylor and Parke-Davis. Tiagabine was generously provided by T. B. Sebree and Abbott Laboratories. W. Bondinell of SmithKline Beecham generously provided the SKF compounds.

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