British Journal of Pharmacology (2002) 135, 93–102

www.nature.com/bjp

Bi-directional transport of GABA in human embryonic kidney (HEK-293) cells stably expressing the rat GABA transporter GAT-1

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1 Bi-directional GABA-transport was studied by performing uptake and superfusion experiments in human embryonic kidney 293 cells stably expressing the rat GABA transporter rGAT-1.

2 K_M and V_{max} values for [^{3}H]-GABA uptake were 11.7 ± 1.8 μM and 403 ± 55 pmol min^{-1} 10^{-6} cells ($n=9$), respectively.

3 Kinetic analysis of outward transport was performed by pre-labelling the cells with increasing concentrations of [^{3}H]-GABA and triggering outward transport with 333 μM GABA. Approximate apparent K_M and V_{max} values were 12 mM and 50 pmol min^{-1} 10^{-6} cells, respectively.

4 GABA re-uptake inhibitors (RI; e.g. tiagabine), as well as, substrates of the rGAT-1 (e.g. GABA, nipecotic acid) concentration dependently decreased [^{3}H]-GABA uptake and increased efflux of [^{3}H]-GABA from pre-labelled cells. The IC_{50} values for inhibiting uptake and the EC_{50} values for increasing efflux were significantly correlated ($r^2=0.99$).

5 On superfusion, RI antagonized the efflux-enhancing effect of the substrates. The effect of the latter was markedly augmented in the presence of ouabain (100 μM), whereas the effect of RI remained unchanged. The most likely explanation for the release enhancing effect of RI is interruption of ongoing re-uptake.

6 The structural GABA-analogue 2,4-diamino-n-butyric acid (DABA) exhibited a bell-shaped concentration response curve on [^{3}H]-GABA efflux with the maximum at 1 mM, and displayed a deviation from the sigmoidal inhibition curve in uptake experiments in the same concentration range. At concentrations below 1 mM, DABA inhibited [^{3}H]-GABA uptake non-competitively, while at 1 mM and above the inhibition of uptake followed a competitive manner.

7 The results provide information of GABA inward and outward transport, and document a complex interaction of the rGAT-1 with its substrate DABA.

British Journal of Pharmacology (2002) **135,** 93–102

Keywords: DABA, 2,4-diamino-n-butyric acid; outward transport; uptake; efflux; superfusion; GABA transport; rat GABA transporter; rGAT-1

Abbreviations: HEK-293 cells, Human embryonic kidney 293 cells; rGAT-1 cells, HEK-293 cells stably expressing the rat GABA transporter GAT-1; RI, re-uptake inhibitors; SDS, sodium dodecyl sulphate

Introduction

Neurotransmitters are stored in presynaptic vesicles and released by exocytosis upon Ca^{2+}-influx into the presynaptic specialization. The signal is terminated by rapid re-uptake of the neurotransmitter by a specific Na^+-dependent transporter into the synaptic terminal or the surrounding glial cells (Nelson, 1998). This general model is applicable to essentially all neurotransmitters, notable exceptions being acetyl choline and nucleotides which are hydrolyzed rather than taken up. Among the neurotransmitters using a transporter for signal termination is GABA (γ-aminobutyric acid), one of the major inhibitory neurotransmitters in the adult mammalian central nervous system; more than 30% of all synapses use GABA as transmitter (Bloom & Iversen, 1971). The inhibition of GABA-transport has become increasingly relevant to clinical pharmacotherapy in the past years. Drugs acting on the GABA-transport (like tiagabine or 2,4-diamino-n-butyric acid; DABA) have proven to act anticonvulsively in animal models (Nielsen *et al*., 1991; Amabeoku & Chikuni, 1993; Taberner & Roberts, 1978; Dalby, 2000). Changes in the number of GABA transporters have been associated with temporal-lobe epilepsy (During *et al*., 1995) and the GABA uptake inhibitor tiagabine is already in use as an antiepileptic drug (Gustavson & Mengel, 1995; Snel *et al*., 1997; Uthman *et al*., 1998). The application of molecular biology to the study of neurotransmitter transporters within the past few years has greatly expanded our knowledge of these molecules and has revealed a far greater complexity of GABA transporters than previously imagined. To date genes for four distinct high-affinity GABA transporters have been cloned: GAT-1, GAT-2, GAT-3 and BGT-1 (Guastella *et al*., 1990; Borden *et al*., 1992; Liu *et al*., 1993 reviewed in Borden, 1996). It is generally assumed that the major physiological function of neurotransmitter transporters is cellular re-uptake of released neurotransmitter; however these transporters are also capable of running

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backwards and releasing neurotransmitter in a Ca^{2+}-independent non-vesicular manner. Carrier-mediated GABA release can be found both on neurons and on glia, induced under the influence of certain drugs, with depolarization or under a decrease in the sodium gradient (Richerson & Gaspary, 1997). It has been proposed that carrier-mediated GABA release can occur *in vivo* during high-frequency neuronal firing and seizures, activating GABA receptors on hippocampal neurones (Gaspary *et al*., 1998), but it can also be observed in the intermediate spiny neurones of the striatum following stimulation by D_1-dopamine receptors (due to a cyclic AMP/PKA-dependent action on the Na^+/K^+-pump, see Schoffelmeer *et al*., 2000). It is common methodology to study neurotransmitter transport in cellular systems containing only the transporter of interest, i.e. uptake and release experiments are performed in cells transfected with the appropriate cDNA: the investigation of reverse transport in these systems, however, has met difficulties resulting from methodological limitations. In a typical experiment of this kind cells are grown in wells loaded with radiolabelled neurotransmitters and the efflux of the radiolabel from the cells is studied by pipetting medium from the well or by measuring the amount of radiolabel in the cells. Although some meaningful results can be generated in this way, the approach is less than ideal. It does not prevent movement of neurotransmitter molecules across the membrane in both directions (uptake and reverse transport at the same time) and it does not allow a satisfactory time resolution of the process under study. A method was developed in our laboratory allowing the superfusion of transfected cells after they have been loaded with radiolabelled neurotransmitter (Pifl *et al*., 1995). This technique has been used successfully to characterize the rat and the human serotonin transporter (Scholze *et al*., 2000; Sitte *et al*., 2000) and has now been applied to study the GABA transporter GAT-1. In most cases superfusion minimizes re-uptake since any released neurotransmitter is washed away immediately by the superfusion buffer.

Here we present data on the uptake and efflux of [^{3}H]-GABA in human embryonic kidney 293 (HEK-293) cells stably expressing the rat GAT-1. Kinetic constants for inward and outward transport are presented and a complex interaction of the rGAT-1 with the substrate 2,4-diamino-n-butyric acid (DABA) is described.

Methods

Cell culture

The cDNA for the rat GABA transporter (GAT-1) was a generous gift of Dr P. Schloss (Zl für seelische Gesundheit, Mannheim, Germany; Sato *et al*., 1995). The coding region was subcloned into pRC/CMV (Invitrogen, Carlsbad, CA, U.S.A.).

For stable expression into HEK-293 cells the same method was used as described in Pifl *et al*. (1996). The stable transfectants (rGAT-1 cells) were grown in Dulbecco's minimal essential medium containing L-analyl-L-glutamine (L-glutamax I™, Gibco Life Technologies, Grand Island NY, U.S.A.), 10% heat-inactivated foetal bovine serum, 50 mg l^{-1} gentamicin and 500 μg ml^{-1} geneticin (G418) on 100 mm-diameter cell culture dishes at 37°C in an atmosphere of 5% CO_2, 95% air.

Uptake

The experiments were performed as described previously (Scholze *et al*., 2000). In brief, 5×10^4 cells were seeded onto poly-D-lysine-coated 48-well plates, and influx was measured 2 days after plating. Each well was washed once with 1 ml of KRH buffer (Krebs-Ringer HEPES buffer; (mM): HEPES 10, NaCl 120, KCl 3, $CaCl_2$ 2, $MgCl_2$ 2, glucose 20, final pH 7.3). Cells were incubated with 10 nM [^{3}H]-GABA (33 Ci $mmol^{-1}$) and various concentrations of unlabelled GABA for 3 min at room temperature. To study drug effects, cells were pre-incubated with the substance of interest for 3 min before adding 10 nM [^{3}H]-GABA and 5 μM unlabelled GABA for another 3 min. Then, the uptake buffer was aspirated, the cells were washed twice with ice-cold buffer and lysed with 0.5 ml of 1% sodium dodecyl sulphate (SDS). Radioactivity in the lysates was measured by liquid scintillation counting. Non-specific uptake was defined as uptake in the presence of 10 μM tiagabine (3 min pre-incubation).

Superfusion

The experiments were performed as described previously (Scholze *et al*., 2000). In brief, cells were grown overnight onto poly-D-lysine coated round glass cover slips (diameter 5 mm) at 4×10^4 cells/well. Cells were loaded with 50 μM [^{3}H]-GABA (0.24 Ci $mmol^{-1}$; 1.2 μCi $well^{-1}$) for 20 min at 37°C in a final volume of 100 μl KRH buffer. The cover slips were then transferred to superfusion chambers (200 μl) and superfused with KRH buffer (0.7 ml min^{-1}) at 25°C. After a washout period of 45 min a stable efflux was obtained and the experiment was started with the collection of 2 min fractions. After three fractions ($t=0$) the cells were exposed to the drug of interest and another five fractions were collected. At the end of the experiment cells were lysed in 1% SDS. Tritium in the superfusate fractions and in the SDS lysates was determined by liquid scintillation counting. The release of [^{3}H] was expressed as fractional rate, i.e. the radioactivity released during a fraction was expressed as a percentage of the total radioactivity present in the cells at the beginning of that fraction. Basal efflux was defined as efflux in the absence of the drug, e.g. mean efflux collected in fractions 1–3. Drug-induced efflux was calculated as the mean value of fractions 6–8 divided by 2 (fraction length = 2 min).

Determination of cell numbers per coverslip and estimation of intracellular GABA concentrations

For the estimation of the number of cells used in the superfusion experiments, 4×10^4 cells were seeded onto round glass coverslips and grown overnight. Coverslips were transferred to superfusion chambers (200 μl) and superfused with KRH buffer (0.7 ml min^{-1}) at 25°C for 45 min. At the end of the superfusion coverslips were removed, washed with KRH lacking glucose, immersed in 60 μl of 1% SDS and the protein content of the cell lysate

was measured (bicinchoninic acid kit, Pierce, Rockford, IL, U.S.A.). Cell numbers were calculated using a standard curve generated on the same day from known amounts of the same cells. This assay was performed in 12 parallel incubations on two different days and yielded a mean cell number of 26,406 ± 2659 per coverslip ($n = 24$). Intracellular drug concentrations were calculated from the accumulated radioactivity in the cells, a cell volume of 1.3 pl (see Sitte *et al*., 2001) and a cell number of 27,000 cells per coverslip.

Data calculation

V_{max}, K_M, EC_{50} and IC_{50} values were calculated using non-linear regression fits performed with Prism (GraphPad, San Diego, CA, U.S.A.). The equation used to estimate K_M and V_{max} values was $Y = V_{max} \times X/(K_M + X)$ where $Y = V$ (picomoles per 10^6 cells per min) and $X =$ substrate concentration (moles per litre).

Chemicals

Tissue culture reagents were from Gibco Life Technologies. [2,3,4-^{3}H]-γ-amino-n-butyric acid ([^{3}H]-GABA) was obtained from ICN (Irvine, CA, U.S.A.). γ-amino-n-butyric acid (GABA), (±)-3-piperidinecarboxylic acid (nipecotic acid), L- and DL-2,4-diamino-n-butyric acid (DABA) and 1β, 3β, 5β, 11α, 14, 19-hexahydroxycard-20[22]-enolide 3-[6-deoxy-α-L-mannopyranoside] (ouabain) were from Sigma-Aldrich Handels GmbH (Vienna, Austria); 1-(2-((diphenylmethylene)amino)oxy)ethyl) - 1,2,5,6 -tetrahydro-3-pyridine-carboxylic acid hydrochloride (NO 711) from RBI (Research Biochemicals International, Natick, MA, U.S.A.). 1,2,5,6-tetrahydropyridine-3-carboxylic acid hydrochloride (guvacine), 1-(4,4-diphenyl-3-butenyl)-3-piperidine-carboxylic acid hydrochloride (SKF 89976A) and 1-[2-[bis[4-(trifluoromethyl)phenyl)methoxy]ethyl] - 1,2,5,6 - tetrahydropyridine - 3 - carboxylic acid hydrochloride (CI 966) were purchased from Tocris Cookson Ltd (Bristol U.K.). R-(−)-1-(4,4-bis(3-methyl-2-thienyl)-3-butenyl-3-piperidine carboxylic acid hydrochloride (tiagabine) was generously provided by Sanofi-Synthelabo (Montpellier, France). All other chemicals were from commercial sources.

Results

HEK-293 cells permanently expressing the rGAT-1 (rGAT-1 cells) exhibited a tiagabine-sensitive, time-, temperature- and concentration-dependent accumulation of [^{3}H]-GABA. Saturation analysis of initial uptake rates revealed a V_{max} value of 403 ± 55 pmol min^{-1} 10^{-6} cells and a K_M value of 11.7 ± 1.8 μM (mean ± s.e.mean of nine independent determinations).

Effects of rGAT-1 substrates and rGAT-1 inhibitors on the efflux and uptake of [^{3}H]-GABA

Basal [^{3}H]-GABA efflux from rGAT cells pre-incubated with [^{3}H]-GABA amounted to 0.062 ± 0.002% min^{-1} or 131.01 ± 9.15 d.p.m. (basal efflux: mean of the three fractions before drug addition, $n = 58$ randomly chosen experiments). Addition of rGAT-1 substrates such as GABA or nipecotic acid or inhibitors of GAT-1 re-uptake such as tiagabine or SKF 89976A to the superfusion buffer caused a concentration and time-dependent increase of [^{3}H]-GABA efflux. Representative effects of GABA and SKF 89976A are shown in Figure 1. Both drugs caused a concentration–dependent increase in efflux that reached a plateau after 6 min of drug exposure with SKF 89976A being only about one-fifth as effective as GABA. Figure 2a shows concentration–response curves for various substrates and re-uptake inhibitors of GAT-1, the resulting EC_{50} values are given in Table 1. The maximum effects on efflux were 0.3% min^{-1} for all substrates except GABA (0.44% min^{-1}) and 0.12% min^{-1} for the re-uptake inhibitors. All drugs were also tested for inhibition of [^{3}H]-GABA uptake: the concentration–response curves are shown in Figure 2b, the corresponding IC_{50} values are given in Table 1. There was a good overall correlation ($r^2 = 0.99$) between the IC_{50} values obtained from uptake experiments

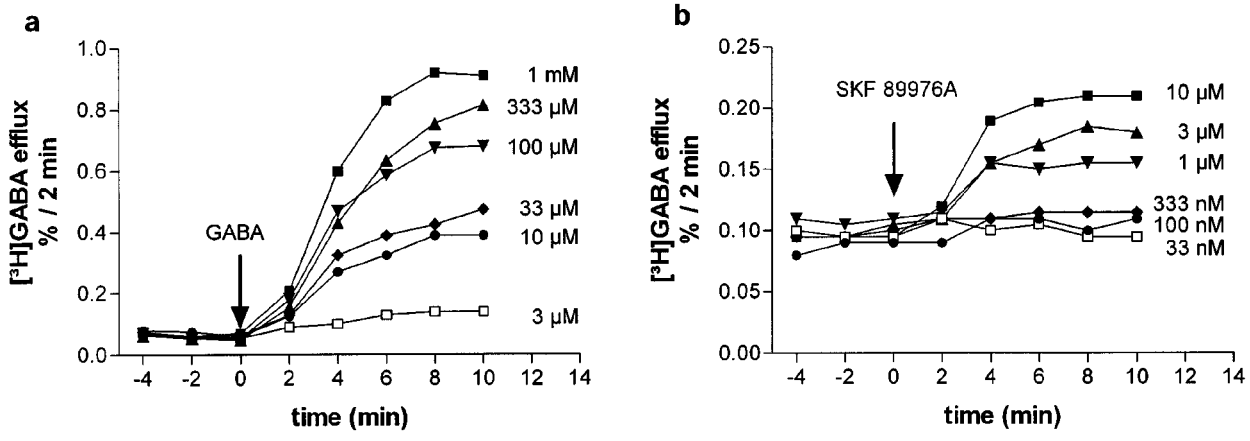

Figure 1 Time course of the effects of GABA and SKF 89976A on [^{3}H]-GABA efflux from HEK-293 cells stably expressing the rGAT-1. rGAT-1 cells were pre-incubated with [^{3}H]-GABA, superfused, and 2 min fractions collected. After three fractions (6 min) of basal efflux, the buffer was switched to a buffer containing different concentrations of GABA (a) or SKF 89976A (b). Data are presented as fractional efflux, i.e. each fraction is expressed as the percentage of radioactivity present in the cells at the beginning of that fraction. Shown is one of three experiments performed in duplicate.

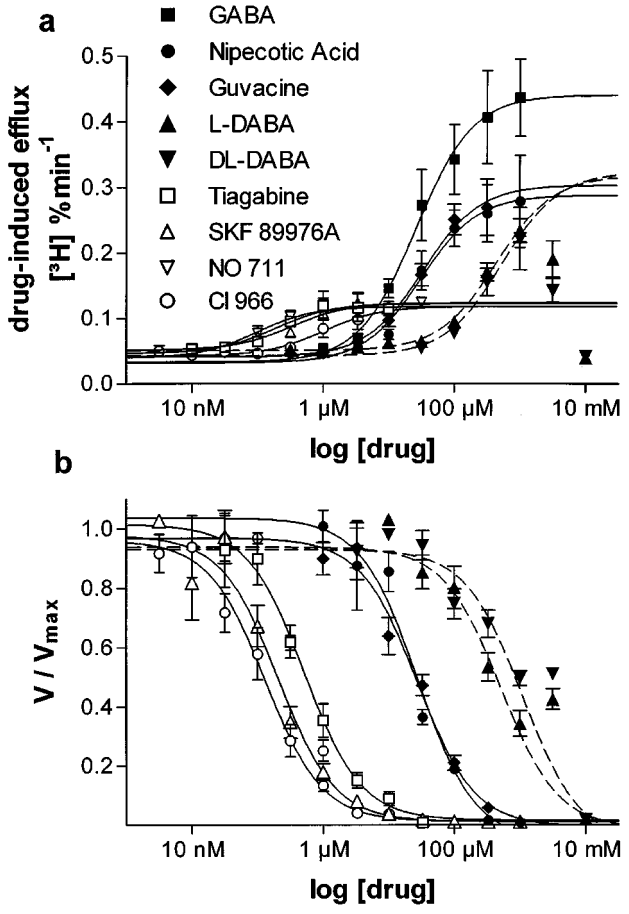

Figure 2 Effect of substrates and re-uptake-inhibitors on uptake and efflux of [^{3}H]-GABA in HEK-293 cells stably expressing the rGAT-1. (a) Concentration–response curves of substrates and re-uptake-inhibitors of GAT-1 for their effects on [^{3}H]-GABA efflux. For experimental details see Figure 1. Drug-induced efflux (% min^{-1}) was calculated as the mean efflux of fractions 6–8 divided by the length of the fraction (=2 min). Symbols represent means±s.e.mean of six observations (three experiments each performed in duplicate). The data were fitted by nonlinear regression. EC_{50} values are given in Table 1. The fits for L-DABA and DL-DABA were done excluding all data points >1 mM (see also Figure 3a). (b) Concentration–inhibition curves of substrates and re-uptake-inhibitors of GAT-1 for their effects on [^{3}H]-GABA uptake. Cells were incubated in 48-well plates (5×10^5 cells/well) with 5 μM [^{3}H]-GABA for 3 min at room temperature as described in Methods. Test drugs were added 3 min before [^{3}H]-GABA using at least six different concentrations. Non-specific uptake was measured in the presence of 10 μM tiagabine. Symbols represent means±s.e.mean of nine observations (three experiments each performed in triplicate). The data were fitted by non-linear regression. IC_{50} values are given in Table 1. The fits for L-DABA and DL-DABA was done excluding all data points >1 mM (see also Figure 3b).

with the EC_{50} values obtained from superfusion experiments (see Table 1).

Effects of DABA on uptake and efflux of [^{3}H]-GABA

With regard to the substrates L-DABA and DL-DABA qualitatively different results were obtained compared to the substrates guvacine or nipecotic acid. The concentration–response relationship for the effect on efflux (Figure 3a) was bell-shaped: efflux enhancement was maximal at 1 mM, diminished at 3 mM and practically absent at 10 mM. Inhibition of [^{3}H]-GABA uptake (Figure 3b) was less pronounced at 3 mM than at 1 mM, but a concentration of 10 mM DABA inhibited uptake completely. In order to

Table 1 IC_{50} and EC_{50} values of rGAT-1 substrates and GABA re-uptake inhibitors for inhibiting [^{3}H]-GABA efflux in rGAT-1 cells

Drug	IC_{50} (μM)	EC_{50} (μM)	IC_{50}/EC_{50}
GABA	27.3±8.7	29.2±0.5	0.94
Nipecotic acid	28.9±2.8	35.5±6.9	0.81
Guvacine	28.1±4.1	27.8±9.1	1.01
L-DABA*	524.7±91.0	507.8±22.7	1.03
DL-DABA*	729.5±125.6	606.7±214.3	1.20
Tiagabine	0.12±0.04	0.16±0.04	0.76
SKF 89976A	0.56±0.19	0.84±0.17	0.67
NO 711	0.17±0.03	0.13±0.01	1.32
CI 966	0.44±0.12	0.94±0.22	0.46

For experimental details see legend to figure 2. *The fits for L-DABA and DL-DABA were done excluding all data points >1 mM (see also Figure 3).

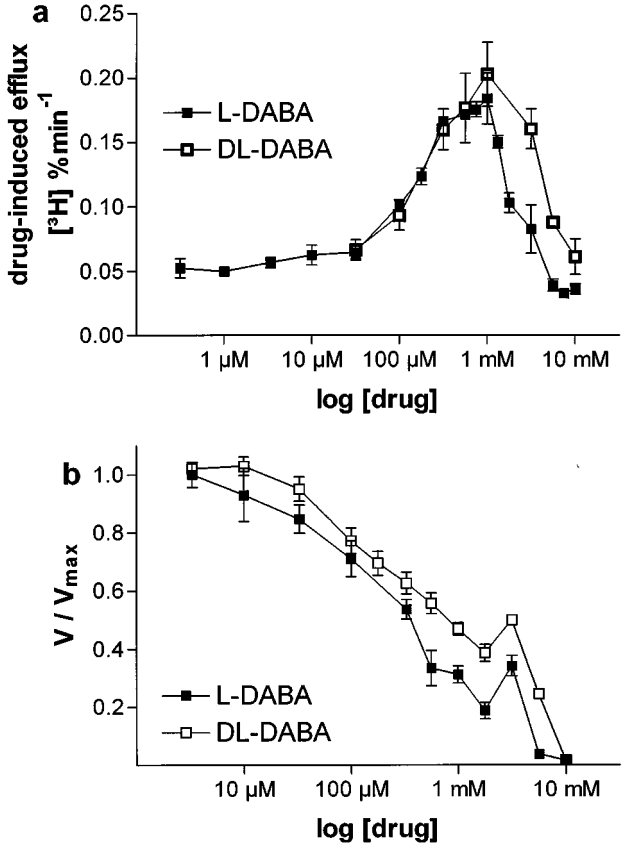

Figure 3 Effect of L- and DL-DABA on uptake and efflux of [^{3}H]-GABA in HEK-293 cells stably expressing the rGAT-1. (a) Concentration–response curve of L- and DL-DABA for their effects on [^{3}H]-GABA efflux. Experimental details see legend to Figure 2. Symbols represent means±s.e.mean of eight observations (four experiments each performed in duplicate). (b) Concentration–inhibition curves of L- and DL-DABA for their effects on [^{3}H]-GABA uptake. Experimental details see legend to Figure 2. Symbols represent means±s.e.mean of 12 observations (four experiments each performed in triplicate).

characterize the biphasic effect of L-DABA on [^{3}H]-GABA uptake more closely, saturation analyses of [^{3}H]-GABA uptake were performed in the absence and presence of various concentrations of L-DABA (Figure 4a). At 100 and 300 μM L-DABA, the K_M of [^{3}H]-GABA uptake remained unchanged, while maximal uptake velocity (V_{max}) declined indicating a non-competitive type of interaction. By contrast, analyses performed with 1, 3 or 4 mM L-DABA revealed a competitive type inhibition with increasing K_M values and a constant V_{max}. These findings show, that at higher concentrations, L-DABA interacts competitively with [^{3}H]-GABA. A similar experiment was performed to study the effect of the substrate nipecotic acid on [^{3}H]-GABA uptake (Figure 4b). Throughout the entire concentration range tested, the V_{max} of [^{3}H]-GABA uptake remained constant, while the K_M value increased; thus, nipecotic acid interacts with [^{3}H]-GABA competitively.

To examine the lack of effect of high concentrations of DABA (1–10 mM) on [^{3}H]-GABA efflux more closely, rGAT-1 cells were superfused and different concentrations of L-DABA were added to the superfusion buffer for a period of 10 min followed by drug-free superfusion for the remainder of the experiment (Figure 5a). At a concentration of 1 mM L-DABA increased [^{3}H]-GABA-efflux which returned to baseline after removal of the drug. By contrast, efflux of [^{3}H]-GABA was stimulated only weakly or not at all after addition of higher concentrations of DABA and increased remarkably after the removal of the drug to a level well above the level attained in the presence of 1 mM DABA, and comparable to the efflux induced with a saturating concentration of nipecotic acid. An additional set of experiments was performed to study the effects of rGAT substrates and RI on GABA induced [^{3}H]-GABA efflux (Figure 5b). rGAT cells were superfused, and a saturating amount of GABA (333 μM) was included for 10 min followed by additional exposure to the test substances at saturating concentrations. The substrate

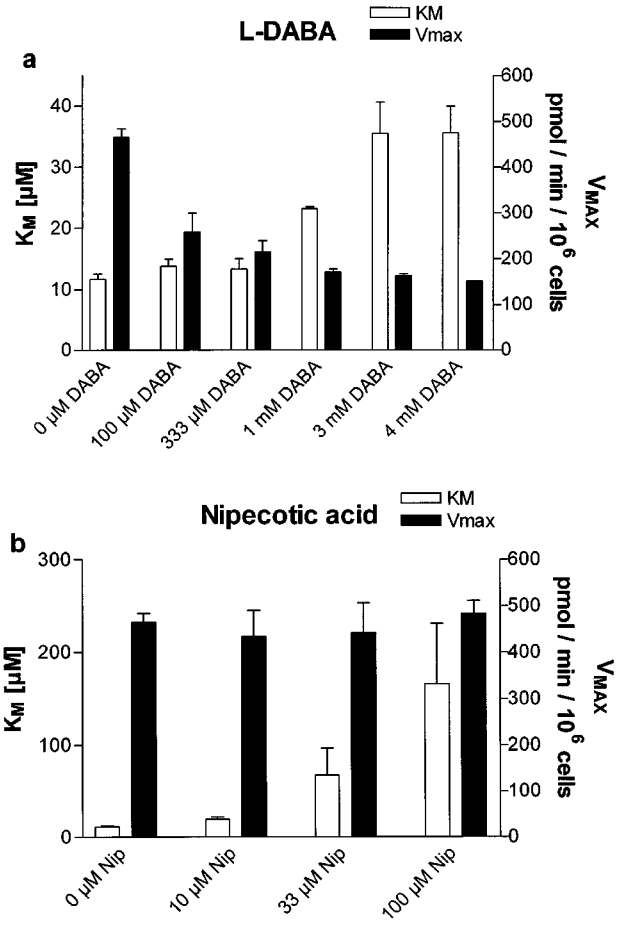

Figure 4 Effect of nipecotic acid and L-DABA on the uptake of [^{3}H]-GABA in HEK-293 cells stably expressing the rGAT-1. Saturation analysis of [^{3}H]-GABA uptake was performed in the presence of different concentrations of L-DABA (a) or nipecotic acid (b). Open bars represent K_M-values obtained (left ordinate), closed bars V_{max}-values (right ordinate). Bars represent means ± s.e.mean of nine observations (three experiments each performed in triplicate).

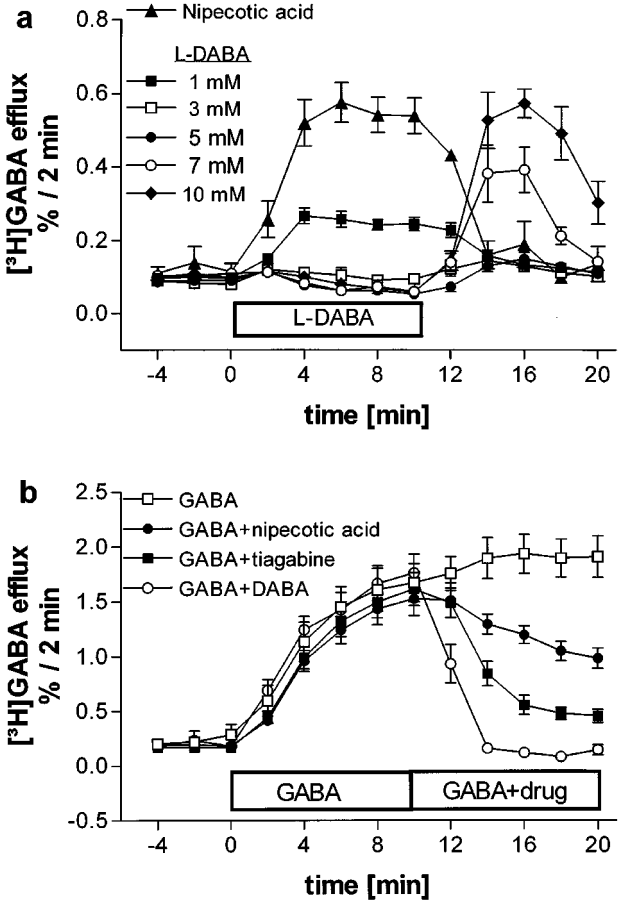

Figure 5 Effects of L-DABA and nipecotic acid on the efflux of [^{3}H]-GABA in HEK-293 cells stably expressing the rGAT-1. (a) Effects on basal efflux of [^{3}H]-GABA. rGAT cells were pre-incubated with [^{3}H]-GABA, superfused, and 2 min fractions collected. L-DABA or nipecotic acid were present from 0 to 10 min. (b) Effects on [^{3}H]-GABA efflux induced by 333 μM unlabelled GABA. rGAT cells were pre-incubated with [^{3}H]-GABA, superfused, and 2 min fractions collected. GABA (333 μM) was added at 0 min and its concentration kept constant throughout the experiment. At 10 minutes different drugs were added to the GABA containing superfusion buffer (nipecotic acid: 33 μM, tiagabine: 10 μM, L-DABA: 10 mM) as indicated by the bar. Data are presented as fractional efflux, i.e. each fraction is expressed as the percentage of radioactivity present in the cells at the beginning of that fraction. Symbols represent means ± s.e.mean of nine observations (one observation = one superfusion chamber; triplicates from three separate experiments).

nipecotic acid (33 μM) decreased GABA-induced [^{3}H]-GABA efflux slightly whereas the RI tiagabine (10 μM), displayed a more marked effect. However, L-DABA at a concentration of 10 mM completely suppressed the GABA effect with efflux reaching basal levels.

Interaction between substrates and blockers of GAT-1

The effect of the potent uptake inhibitor tiagabine on GABA-induced [^{3}H]-GABA release was analysed. After a 10-min exposure to different concentrations of tiagabine (0 to 33 μM) GABA (333 μM) was added to the superfusion buffer for additional 10 min. As shown in Figure 6, tiagabine alone increased [^{3}H]-GABA efflux in a concentration–dependent manner, but likewise inhibited further efflux induced by GABA. At tiagabine concentrations of more than 33 μM the release-enhancing action of 333 μM GABA was abolished.

Effect of Na^+, K^+-ATPase inhibition in superfusion experiments

A series of experiments was performed in which the cells were exposed to ouabain, a measure which is expected to raise the intracellular sodium concentration and to facilitate outward transport of substrate. When ouabain (100 μM) was included in the superfusion buffer during the 45-min washout period, the efflux rate at the beginning of superfusate collection was nearly twice as high as compared to ouabain-free conditions ($0.083 \pm 0.006\%$ min^{-1} versus $0.052 \pm 0.003\%$min^{-1}, $n=20$). [^{3}H]-GABA efflux induced by any substrate (including DABA) was massively enhanced in the presence of ouabain, while the efflux-enhancing effect of re-uptake inhibitors was not changed. Figure 7 shows representative experiments with GABA and SKF 89976A induced efflux.

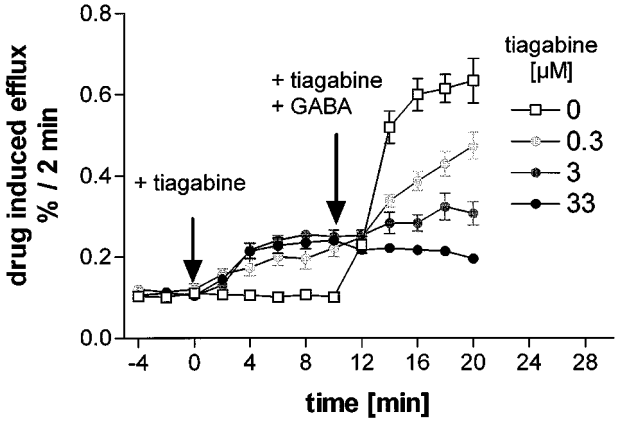

Figure 6 Interaction of tiagabine and GABA on [^{3}H]-GABA efflux from HEK-293 cells expressing the rGAT-1. HEK-293 cells permanently expressing the rGAT-1 were pre-incubated with [^{3}H]-GABA, superfused and 2 min fractions were collected. After three fractions of basal efflux (6 min; $t=0$), cells were exposed to buffers containing different concentrations of tiagabine (0.3 μM: light grey; 3 μM: dark grey; 33 μM: black) or left at control conditions (open squares). After 10 min GABA (333 μM) was added to all superfusion channels. Data are presented as fractional efflux, i.e. each fraction is expressed as the percentage of radioactivity present in the cells at the beginning of that fraction. Symbols represent means $\pm$ s.e.mean of six observations (one observation = one superfusion chamber; triplicates from two separate experiments).

Comparison of inward and outward transport rates

A series of experiments was performed, in which initial uptake rates (= Inward Transport Rates) and Outward Transport Rates were determined in parallel. One representative set of experiments is shown in Figure 8. The uptake experiment in Figure 8a rendered a K_M of 10.8 μM and a V_{max} of 335 pmol min^{-1} 10^{-6} cells. In parallel, an efflux experiment was done by pre-incubating the cells with different concentrations of [^{3}H]-GABA (37°C, 20 min) and inducing efflux with unlabelled GABA (333 μM) or tiagabine (10 μM). Maximal efflux values were determined by averaging

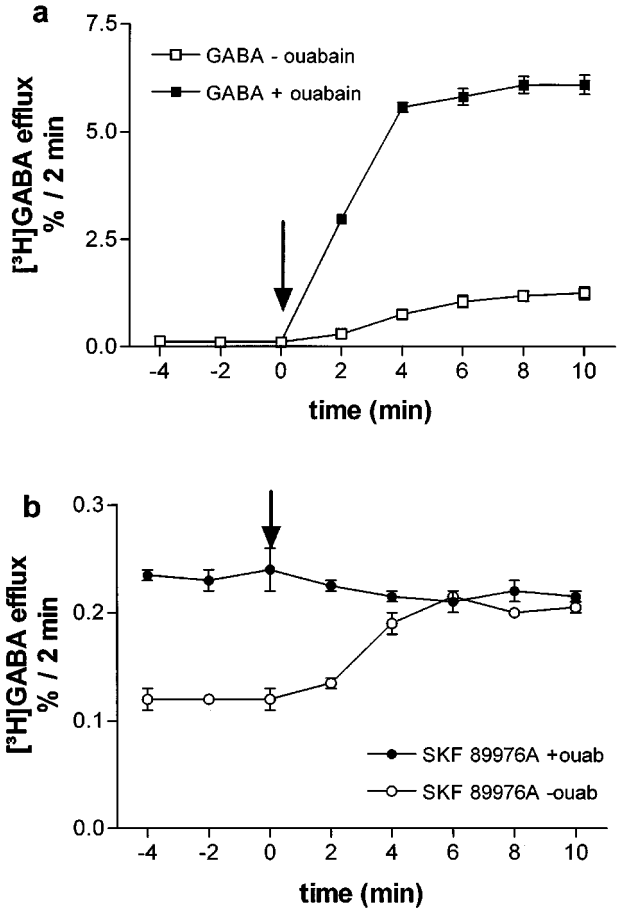

Figure 7 Influence of Na^+, K^+-ATPase inhibition on the effects of GABA and SKF 89976A on [^{3}H]-GABA efflux from HEK-293 cells expressing the rGAT-1. (a) GABA induced efflux in the absence or presence of ouabain. HEK-293 cells permanently expressing the rGAT-1 were preincubated with [^{3}H]-GABA and superfused with KRH buffer (open squares) or ouabain (100 μM; closed squares) for 45 min. Then the collection of 2 min fractions was started. After three fractions (6 min, $t=0$) cells were exposed to a buffer containing 333 μM GABA. (b) SKF 89976A induced efflux in the absence and presence of ouabain: HEK-293 cells permanently expressing the rGAT-1 were pre-incubated with [^{3}H]-GABA and superfused with KRH buffer (open circles) or KRH buffer containing 100 μM ouabain (closed circles) for 45 min. Then the collection of 2 min fractions was started. After three fractions (6 min, $t=0$) cells were exposed to a buffer containing 10 μM SKF 89976A. All data are presented as fractional efflux, i.e. each fraction is expressed as the percentage of radioactivity present in the cells at the beginning of that fraction. Symbols represent means $\pm$ s.e.mean of four observations (one observation = one superfusion chamber; duplicates from two separate experiments).

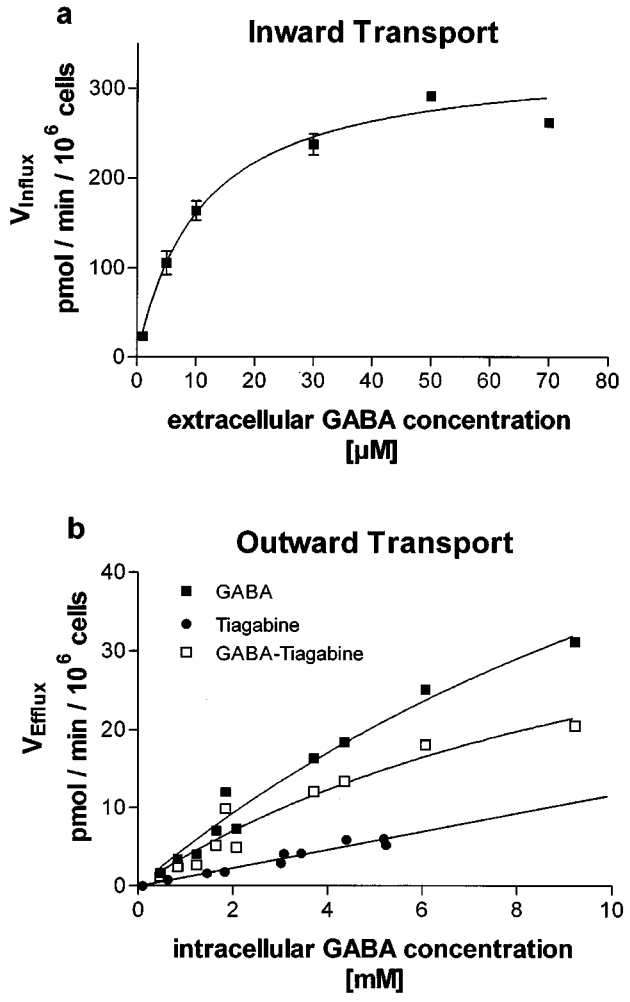

Figure 8 Inward and outward transport of [^{3}H]-GABA. (a) Inward Transport: HEK-293 cells stably expressing the rGAT-1 were incubated with different concentrations of [^{3}H]-GABA for 3 min. Non-specific uptake was defined as uptake in the presence of 10 μM tiagabine. Non-linear fitting was used to calculate V_{max} and K_M. (b) Outward Transport: HEK-293 cells stably expressing the rGAT-1 were grown on 5-mm coverslips, pre-incubated with different concentrations of [^{3}H]-GABA (3–50 μM), superfused, challenged with 333 μM GABA or 10 μM tiagabine using the same protocol as given in Figure 1. Efflux rates (V_{efflux}; pmol min^{-1} 10^{-6} cells) were calculated from the mean value of fractions 3–5 after drug addition, with a number of 27,000 cells / coverslip and an intracellular volume of 1.3 pl (see Methods). Specific efflux rates (open squares) were calculated from total GABA-induced efflux (closed squares) minus diffusion-based efflux estimated from the linear regression line of the V_{efflux} values for tiagabine (closed circles). Non-linear fitting was performed as in panel (a).

the data points between 4 and 10 min after drug addition and plotting against the estimated intracellular substrate concentration (Figure 8b). Since the effect caused by tiagabine is supposed to be due to interruption of high affinity re-uptake of GABA leaving the cell by diffusion, the GABA effect was corrected for the tiagabine-induced efflux to obtain transporter mediated release (open squares in Figure 8b). The intracellular concentration of [^{3}H]-GABA at which the half-maximal rate of reverse transport was reached (K_M for reverse transport) was 12 mM, the maximal velocity for reverse transport was about 50 pmol min^{-1} 10^{-6} cells.

Discussion

The aim of the present study was to characterize bi-directional GABA transport in cells stably expressing the rGAT-1. For this purpose the effects of re-uptake inhibitors as well as substrates of GAT-1 were studied in uptake and superfusion experiments. While inward transport is easily monitored in uptake assays, outward transport is more difficult to measure as an isolated process. Cells have to be pre-incubated with radiolabelled substrate before outward transport can be induced, and released substrate may be recaptured by ongoing inward transport. For this reason superfusion experiments were performed which rest on the premise that the flow rate of the buffer is too rapid to permit re-uptake of released transmitter (Raiteri *et al*., 1974) and thus allow to measure isolated outward transport. When [^{3}H]-GABA labelled cells were exposed to substrates of the rGAT-1 (GABA, nipecotic acid, guvacine, DABA) under superfusion conditions all substances clearly caused outward transport as evidenced by an increase in tritium efflux into the superfusion medium. The most active compound proved to be the natural substrate GABA which caused an increase by about a factor of 7 as compared to baseline efflux. The other transporter substrates were clearly less effective. A similar result, at least with respect to nipecotic acid, has been observed in CHO cells expressing the rGAT-1 (Corey *et al*., 1994). It is assumed that substrates such as nipecotic acid may bind tightly to the GABA binding site but after transport to the intracellular compartment may dissociate slowly from the transporter. This would prevent the binding of intracellular GABA and consequently reduce its outward transport. When well-known inhibitors of GABA uptake were tested in the superfusion experiments, they also caused a small increase in efflux from about 0.06% min^{-1} basal to 0.12% min^{-1} in the presence of drugs. This is in line with similar findings in other studies, including our own. For instance, a distinct efflux enhancing effect of re-uptake inhibitors was seen in experiments using rotating disc electrode voltammetry in cells expressing the human norepinephrine transporter (Chen *et al*., 1998; Chen & Justice, 1998) or in superfusion experiments on serotonin transporter expressing cells pre-incubated with [^{3}H]-serotonin (Scholze *et al*., 2000; Sitte *et al*., 2000). The most likely explanation for the observation was drug-induced interruption of ongoing re-uptake of substrate that is leaving the cell by diffusion. This seems also true for the present experiments considering that (1) the intracellular concentration of [^{3}H]-GABA is in the millimolar range (Figure 8b), (2) the affinity for GABA inward transport is high, and (3) the expression level of the rGAT-1 is high (Scholze *et al*., 2001).

Moreover, exposure of the cells to ouabain, a measure known to cause a rise in the sodium concentration at the inside of the plasma membrane and a decrease in K_M value for outward transport (Raiteri *et al*., 1978; Bönisch, 1986; Liang & Rutledge, 1982), resulted in a massive enhancement of [^{3}H]-GABA efflux induced by all transporter substrates, but not of the efflux induced by RI's. This finding strongly argues against the possibility that efflux induced by re-uptake inhibitors is a consequence of reversed transport and confirms the previous interpretation of drug induced interruption of re-uptake.

The range of IC_{50} values obtained in the uptake experiments was 0.1–700 μM, with individual values in very good agreement with the data of Borden *et al.* (1994) in rGAT-1 transfected LM(tk$^-$) cells. Clark *et al.* (1992) reported a similar rank order of potencies in HeLa cells transiently expressing rGAT-1, though the absolute potencies were about 10 to 30 times lower than those presented here (Table 1). One reason for the difference may be the use of transient transfections by Clark *et al.* (1992) as opposed to permanent transfection in the present study (for review see Borden, 1996). Other possibilities are the use of different temperatures (37°C vs 25°C) in the two studies.

A comparison of IC_{50} values obtained in uptake experiments with the EC_{50} values obtained in superfusion experiments showed a good correlation, the ratios of IC_{50}/EC_{50} being about unity for almost all substances (Table 1). This result is expected for uptake-inhibitors, as they exert the same effect (block of re-uptake) in both types of experiments. It is not as obvious for the substrates which induced outward transport in the same concentration range as they inhibited inward transport. In fact, a similar analysis in the rat serotonin transporter consistently yielded IC_{50}/EC_{50} ratios of >2 (up to 10; Sitte *et al.*, 2000), i.e. all transporter substrates acted as preferential releasers. This obviously does not hold true for rGAT-1 substrates.

A noticeable finding of the present study concerns the effects of 2,4-diamino-n-butyric acid (DABA), a close structural analogue of GABA. The concentration–response curve of DABA for inducing efflux of radioactivity from [^{3}H]-GABA labelled cells was clearly bell-shaped with a maximum at 1 mM. Interestingly, at the same concentration of DABA a deviation from its concentration–response curve was seen when the compound was used to inhibit GABA uptake (Figure 3). A more detailed analysis revealed two types of interaction: L-DABA inhibited [^{3}H]-GABA uptake in an allosteric manner at drug concentrations below 1 mM, while at 1 mM or above the interaction was clearly competitive. By comparison, nipecotic acid inhibited [^{3}H]-GABA uptake competitively at all concentrations tested, which is the expected type of inhibition for a transporter substrate, that is transported by a similar mechanism as GABA. However, at low concentrations L-DABA behaves like a substrate, too: DABA induces efflux to a similar extent as other substrates do, although maximal efflux can not be reached (see Figure 2). But results from Figure 5 show (under certain conditions) DABA induced efflux to be as high as that elicited by nipecotic acid. In addition, DABA induced [^{3}H]-GABA efflux can be augmented significantly by pretreating the cells with ouabain (data not shown), which is a clear indication for substrate induced transporter mediated release. In contrast to nipecotic acid, DABA has an allosteric effect on [^{3}H]-GABA uptake mediated by rGAT-1 at the very same concentration release is caused. The observed results lead to the conclusion that DABA is transported by GAT-1 through a different mechanism than GABA, otherwise the interaction would also be of the competitive type. In fact, it is known from work in synaptosomes that GABA and DABA transport differ in several instances such as the requirements for chloride and potassium (Roskoski, 1981), the dependence on external sodium (Simon & Martin, 1973; Weitsch-Dick *et al.*, 1978), or the co-transport of sodium (Erecinska *et al.*, 1986). Therefore, a complex interaction at the rGAT-1 seems possible.

However, this behaviour of DABA has so far not been described in other experimental systems. Corey *et al.* (1994) studied inhibition by DABA of GABA uptake in rGAT-1 expressing CHO cells, but did not observe a deviation in the probit analysis. The reason for this result is most likely the use of lower DABA concentrations (<0.5 mM) than those used in the present study. However, it is a well known fact, that DABA is able to inhibit GABA uptake (Iversen, 1971) and to stimulate release of the neurotransmitter (Simon *et al.*, 1974).

Interestingly, a bell-shaped concentration–response curve for outward transport induced by a transporter substrate is not unique for the rGAT-1. Wall *et al.* (1995) reported bell-shaped curves for various amphetamines in LLC-PK1 cells expressing the dopamine, norepinephrine or serotonin transporter. In addition, Pifl *et al.* (1999) described a very similar behaviour of amphetamine at the norepinephrine-transporter expressed in HEK cells: the drug increased efflux up to a concentration of 1 μM but suppressed it completely at 100 μM. The authors proposed that amphetamine might interact in two different ways with the norepinephrine-transporter: on the one hand as substrate transported into the cell, which is required for its releasing action, on the other hand extracellularly, by blocking with low affinity the translocation of substrate in both directions. A similar situation may apply to the present experiments and would account for the observation that no increase in efflux of [^{3}H]-GABA was observed when high concentrations of DABA entered the superfusion chamber, but efflux promptly occurred after a switch to drug-free buffer (Figure 5a): in the former situation, DABA may block the transporter by interaction with the transport-blocking extracellular site, in the latter, DABA would first be removed from that site and then could trigger outward transport. In line with this idea are the effect sizes in the two types of experiments. The dotted lines in Figure 2 are fits of the effects of racemic DABA and L-DABA using only the data points up to the concentration of 1 mM. The fit indicates a maximum of the same magnitude as with nipecotic acid or guvacine. However, because of the uptake-inhibiting effect, the estimated maximum is not reached and a bell-shaped concentration–response curve results. On the other hand, when the experiments were performed by first introducing a high concentration of DABA which is subsequently washed away (Figure 5a), the effect on outward transport reached exactly the same maximum as that of nipecotic acid (guvacine not tested in this experiment). It is conceivable that during exposure of the cells to the high concentration enough DABA entered the cells by diffusion to induce outward transport once the extracellular block is released. The findings of Erecinska *et al.* (1986) may also be of note in this context. The authors studied DABA transport in rat brain synaptosomes and suggested a very close proximity of the amino acid (DABA) binding site and one of the sodium binding sites. The unique nature of DABA's action at the rGAT-1 is also apparent from the results in Figure 5b. When outward transport of [^{3}H]-GABA was brought to a maximum using a saturating concentration of unlabelled GABA and then inhibited by the addition of an RI or substrates, DABA exerted the strongest effect, bringing efflux down to basal

levels, even lower than tiagabine. Taken together, the present observations support the notion that the interaction of DABA with the rGAT-1 is different to the interaction of other drugs targeting this transporter.

In a final series of experiments transport rates of uptake and reverse transport were compared. Our cells apparently accumulate substrate up to millimolar concentrations which is in the same range of values as described in other studies with non-neuronal cell lines expressing neurotransmitter transporters (Chen & Justice, 1998; Sitte *et al*., 2001). The relationship between the estimated intracellular concentrations of [^{3}H]-GABA and rates of GABA-evoked [^{3}H]-GABA efflux displayed saturation kinetics with an apparent K_M value of approximately 1000 fold the value found for inward transport (10.8 μM vs 12 mM, Figure 8). This is again comparable to results in other cells, e.g. 0.88 μM versus 396 μM for dopamine at the human norepinephrine transporter (Chen & Justice, 1998) or 0.60 μM vs 564 μM and 17 μM versus 7 mM for serotonin and MPP^+, respectively, at the human serotonin transporter (Sitte *et al*., 2001). The marked difference in the K_M value for inward and outward transport may be caused by several factors. Mabjeesh & Kanner (1989) studied GABA transport in inverted membrane vesicles from rat brain and noted that transport from the cytosolic side to the exterior had a 100 fold higher K_M than transport from the exterior to the cytoplasm. Moreover, an increase in K_M of substrate transport at low sodium concentrations is a well known phenomenon at other sodium- and chloride-dependent transporters (Graefe & Bönisch, 1989).

In conclusion, the present report provides corroborative evidence that outward transport of GABA by the rGAT-1 can readily be initiated by transporter substrates while uptake inhibitors do not exert this effect. Furthermore, data on quantitative aspects of inward and outward transport are provided and the complex interaction of DABA with the transporter is highlighted.

This work was supported by the Austrian Science Foundation, project P-14509.

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(Received October 8, 2001
Revised October 15, 2001
Accepted October 17, 2001)