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Vigabatrin transport across the human intestinal epithelial (Caco-2) brush-border membrane is *via* the H^+-coupled amino-acid transporter hPAT1

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1 The aim of this investigation was to determine if the human proton-coupled amino-acid transporter 1 (hPAT1 or SLC36A1) is responsible for the intestinal uptake of the orally-administered antiepileptic agent 4-amino-5-hexanoic acid (vigabatrin).

2 The Caco-2 cell line was used as a model of the human small intestinal epithelium. Competition experiments demonstrate that [^{3}H]GABA uptake across the apical membrane was inhibited by vigabatrin and the GABA analogues *trans*-4-aminocrotonic acid (TACA) and guvacine, whereas 1-(aminomethyl)cyclohexaneacetic acid (gabapentin) had no affect.

3 Experiments with 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF)-loaded Caco-2 cells demonstrate that apical exposure to vigabatrin and TACA induce comparable levels of intracellular acidification (due to H^+/amino-acid symport) to that generated by GABA, suggesting that they are substrates for a H^+-coupled absorptive transporter such as hPAT1.

4 In hPAT1 and mPAT1-expressing *Xenopus laevis* oocytes [^{3}H]GABA uptake was inhibited by vigabatrin, TACA and guvacine, whereas gabapentin failed to inhibit [^{3}H]GABA uptake.

5 In Na^+-free conditions, vigabatrin and TACA evoked similar current responses (due to H^+/amino-acid symport) in hPAT1-expressing oocytes under voltage-clamp conditions to that induced by GABA (whereas no current was observed in water-injected oocytes) consistent with the ability of these GABA analogues to inhibit [^{3}H]GABA uptake.

6 This study demonstrates that hPAT1 is the carrier responsible for the uptake of vigabatrin across the brush-border membrane of the small intestine and emphasises the therapeutic potential of hPAT1 as a delivery route for orally administered, clinically significant GABA-related compounds.
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Abbreviations: AED, anti-epileptic drugs; AHA, 4-amino-5-hexynoic acid or γ-acetylenic GABA; BCECF, 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein; BBB, blood–brain barrier; BBMV, brush-border membrane vesicles; GABA, γ-aminobutyric acid; gabapentin, 1-(aminomethyl)cyclohexaneacetic acid; GABA-T, GABA-transaminase; HRPE, human retinal pigment epithelia; MCT, monocarboxylate transporter; OAT, organic anion transporter; OCT, organic cation transporter; PAT, H^+-coupled amino acid transporter; PepT1, H^+-coupled di/tripeptide transporter; pH_i, intracellular pH; TACA, *trans*-4-aminocrotonic acid; vigabatrin, 4-amino-5-hexanoic acid or γ-vinyl GABA

Introduction

The initial absorption barrier for any orally delivered agent is the brush-border membrane of the human small intestinal epithelium. The transmembrane transport of many nutrients and drug molecules across this membrane occurs *via* carrier-mediated transport mechanisms which can be Na^+-coupled, H^+-coupled or ion-independent. Previously, we have characterised, using the human intestinal epithelial cell line Caco-2 grown as confluent monolayers on permeable filters, a H^+-coupled amino-acid transporter (system PAT) which is localised specifically to the apical membrane of these human enterocytes (Thwaites *et al*., 1993b; 1995c; Chen *et al*., 2003). System PAT transports a broad spectrum of substrates including small zwitterionic amino acids (alanine, proline and glycine) (Thwaites *et al*., 1993b; 1995c), β-amino acids (β-alanine and taurine) (Thwaites *et al*., 1993a; Thwaites & Stevens, 1999), osmolytes (betaine) (Thwaites *et al*., 1995c; Boll *et al*., 2003), proline analogues (including L-azetidine-2-carboxylic acid, *cis*-4-hydroxy-L-proline and 3,4-dehydro-D, L-proline) (Metzner *et al*., 2004) and a number of orally delivered neuromodulatory agents including D-serine (used in the treatment of schizophrenia) (Thwaites *et al*., 1995a, c; Tsai *et al*., 1998), D-cycloserine (used as an orally delivered antibiotic and in the treatment of schizophrenia) (Ranaldi *et al*., 1994; Thwaites *et al*., 1995a; 2000; Evins *et al*., 2002) and

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γ-aminobutyric acid (GABA) and GABA analogues (including nipecotic acid and isonipecotic acid) (Thwaites *et al*., 2000; Metzner *et al*., 2004). The cDNA encoding the human proton-coupled amino-acid transporter (hPAT1, *SLC36A1*) was cloned from a Caco-2 cell cDNA library and, when expressed in isolation in heterologous cell types (*Xenopus laevis* oocytes or human retinal pigment epithelial (HRPE) cells), displays the same functional properties and substrate specificity as the endogenous system PAT (namely pH-dependent, Na^+-independent, low-affinity, high-capacity transport) (Thwaites *et al*., 1993a; 1995c; Chen *et al*., 2003).

System PAT-like (Na^+-independent, pH-dependent) amino-acid transport has also been demonstrated in rat small intestine (Anderson *et al*., 2004). Immunofluorescence studies, using a PAT1-specific antibody, localised PAT1 exclusively to the brush-border membrane of Caco-2 cell monolayers, and rat and human small intestine (Chen *et al*., 2003; Anderson *et al*., 2004). System PAT (and the hPAT1 clone) represent the high-capacity *imino acid carrier*, which has been described in rat small intestine over several decades and which has an identical substrate specificity to system PAT/hPAT1 (Munck, 1966; Munck *et al*., 1994; Anderson *et al*., 2004). The direct link between PAT1 and the imino acid carrier was not made until recently because of the apparent differences in ion dependency between studies in oocytes (where transport is Na^+-independent) compared with those in intact epithelia, for example, rat small intestine or Caco-2 cell monolayers (where transport can be Na^+-dependent). The apparent Na^+-dependence of this H^+-driven transport system (system PAT/PAT1/imino acid carrier) in intact epithelia is due to a functional coupling with the Na^+/H^+ exchanger NHE3 to maintain the H^+-electrochemical gradient during transport, which is required for optimal absorption of PAT1 substrates to occur (Thwaites *et al*., 1999; Anderson *et al*., 2004; Anderson & Thwaites, 2005). The presence of this multifunctional absorptive transport system in the small intestine, where the H^+-gradient is relevant physiologically due to the 'acid microclimate' at the mucosal surface (Rawlings *et al*., 1987; McEwan *et al*., 1988; Daniel *et al*., 1989), provides a potential route for nutrient, osmolyte and drug absorption.

The neutral amino acid GABA acts as the principal inhibitory neurotransmitter in the mammalian central nervous system (Patsalos, 1999). A decrease in GABA levels, and thus perturbations in overall brain activity, is associated with the etiology of numerous neurological disorders such as anxiety, pain and epilepsy (reviewed in Wong *et al*., 2003). Several pharmacological approaches to modulating GABAergic function have been investigated in humans including direct activation of the GABA-receptor by specific agonists (Krogsgaard-Larsen, 1981), inhibition of rapid GABA uptake by both neuronal and glial cell bodies (Krogsgaard-Larsen *et al*., 1987; Krogsgaard-Larsen, 1988) or inhibition of GABA metabolism (Bolton *et al*., 1989).

Owing to the central inhibitory nature of GABAergic neurotransmission and its relationship with neurological disorders, the ability to deliver orally, clinically-active, GABA-related agents is of significant therapeutic interest. One close structural analogue of GABA is 4-amino-5-hexanoic acid or γ-vinyl GABA (vigabatrin, (brand name Sabril, Aventis)). Vigabatrin binds covalently to the active site of GABA-transaminase (GABA-T), the enzyme responsible for the catabolism of GABA. Binding irreversibly, vigabatrin inactivates GABA-T in a concentration-dependent manner and subsequently induces a universal increase in extracellular GABA levels in the brain (Preece *et al*., 1994; Petroff *et al*., 1996). Vigabatrin has been used extensively as adjunctive treatment for partial epilepsy and is also effective as a monotherapy for treatment of infantile spasms (West syndrome), although use is now restricted due to irreversible visual field defects associated with treatment (Patsalos & Duncan, 1994; Patsalos, 1999; Hadjikoutis *et al*., 2005).

Gabapentin (1-(aminomethyl)cyclohexaneacetic acid (brand name Neurontin, Pfizer)), also a synthetic analogue of GABA, is used clinically as adjunctive therapy for partial seizures (Bryans & Wustrow, 1999). Its mode of action remains unclear. However, it has been suggested that binding to $GABA_B$ receptors negatively coupled to voltage-dependent calcium channels may account for the anticonvulsant properties of gabapentin (Bertrand *et al*., 2001).

Following oral administration, both vigabatrin and gabapentin are rapidly absorbed with bioavailabilities of 80–90% and 60%, respectively (Loscher *et al*., 1993; Patsalos, 1999). It has been suggested that gabapentin absorption is Na^+-independent and may be mediated by a L-type amino-acid transporter and/or system $b^{0,+}$ (Stewart *et al*., 1993; Piyapolrungroj *et al*., 2001; Uchino *et al*., 2002). However, the route of oral absorption of vigabatrin remains undetermined. We have demonstrated clearly that hPAT1 is the nutrient carrier involved in the absorptive transport of GABA across the apical surface of the human small intestinal epithelium (Thwaites *et al*., 2000; Anderson *et al*., 2004). The purpose of this investigation was to determine if hPAT1 is responsible for the transport of these GABA-related therapeutic agents across the intestinal brush-border membrane, which is an essential stage in facilitating the high oral bioavailability of many clinically administered, anticonvulsant agents. In order to determine the structural limitations of hPAT1 for transport of antiepileptic drugs (AED), other known GABA analogues that are not used clinically (including *trans*-4-aminocrotonic acid (TACA) and guvacine) were included for comparison. Identification of the route of oral absorption and the nature of the nutrient carriers involved (if any) in the intestinal transport of vigabatrin and gabapentin could prove important for future antiepileptic drug design.

Methods

Materials

4-Amino-*n*-[2,3-^{3}H]butyric acid (GABA) (81 Ci mmol^{-1}), L-[2,3-^{3}H]proline (43 Ci mmol^{-1}), [2-^{3}H]glycine (16 Ci mmol^{-1}) and D-[^{14}C]mannitol (59 mCi mmol^{-1}) were from Amersham Biosciences (Little Chalfont, U.K.). [3-^{3}H(*N*)β-Alanine (50 Ci mmol^{-1}) was from American Radiolabeled Chemicals (St Louis, U.S.A.). [^{14}C]Gly-Sar (56.7 mCi mmol^{-1}) was from Cambridge Research Biochemicals (Stockton-on-Tees, U.K.). The acetoxy-methyl ester of 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) was from Invitrogen Ltd. (Paisley, U.K.). Cell culture plasticware was from Corning-Costar Ltd. (High Wycombe, U.K.) and all cell culture media and supplements were from Sigma-Aldrich Ltd. (Poole, U.K.). Vigabatrin, gabapentin, guvacine and TACA were all purchased from Tocris (Avonmouth, U.K.). FastTrack 2.0

Kit and TOPO TA Cloning kit were from Invitrogen. Omniscript Reverse Transcriptase was from Qiagen (Crawley, U.K.). Expand High Fidelity PCR System was purchased from Roche Diagnostics (Lewes, U.K.) and mMESSAGE mMACHINE Kit was from Ambion (Huntingdon, U.K.). All other chemicals were from VWR Ltd./Sigma-Aldrich (Poole, U.K.) and were of the highest quality available.

Apical amino acid uptake in Caco-2 cell monolayers

Caco-2 cells (passage number 108–124) were cultured and prepared as confluent monolayers, as described previously (Thwaites *et al*., 1993a, b; 2000). Cell confluence was estimated by microscopy and determination of transepithelial electrical resistance (R_T) measured at 37°C. [^{3}H]GABA (0.5 μCi ml^{-1}; 20–100 μM) uptake across the apical membrane of Caco-2 cell monolayers was measured over 5–15 min at 37°C, as described previously (Thwaites *et al*., 1993a, b; 2000). Uptake was measured in the absence of external Na^+, at an apical pH of 5.5 (basolateral pH 7.4) and in the presence/absence of various compounds (see figure legends for details). Cell monolayer-associated radiolabel was determined by scintillation counting. Transepithelial apical-to-basal (J_{a-b}) [^{3}H]GABA (0.5 μCi ml^{-1}, 20 μM) transport was measured over 90 min in Na^+-free conditions (apical pH 5.5 or pH 7.4, basolateral pH 7.4) and at apical pH 5.5 in the presence of cold GABA, vigabatrin, isonipecotic acid and nipecotic acid (all 10 mM). [^{14}C]Mannitol (0.5 μCi ml^{-1}, 20 μM) was used to estimate passive paracellular transport under all experimental conditions tested.

Intracellular pH (pH_i) measurements

Caco-2 cell monolayers were loaded with the pH sensitive dye BCECF, and changes in pH_i were measured by microspectrofluorimetry, as described previously (Thwaites *et al*., 1993a, b; 2000). The ability of GABA and GABA analogues (10 mM) to induce intracellular acidification in Caco-2 cell monolayers was tested by addition of each compound (at pH 5.5, Na^+-free) to either the apical bathing solution (with basolateral pH 7.4, Na^+-free) or the basolateral bathing solution (with apical pH 7.4, Na^+-free). All solutions were preheated to 37°C. The rate of change of intracellular pH ($\Delta pH_i min^{-1}$), due to a change in the composition of the superfusate, was calculated by linear regression (Photon Counter System 4.7, Newcastle Photometric Systems, U.K.) by comparison of the linear portions of the trace over 30–50 s (15–25 data points) periods before and after the change in composition (Thwaites *et al*., 2000). Example traces are presented as a change in intracellular pH (ΔpH_i) over time.

Molecular cloning and in vitro *transcription of hPAT1*

Total RNA was isolated from Caco-2 cells using FastTrack 2.0 Kit (Invitrogen). RNA (1 μg) was used to synthesise cDNA with random hexamer oligonucleotides (1 μM) and Omniscript RT (Qiagen). The gene specific oligonucleotide primers for PCR (5′-CAGATGCTCCAGCTG-3′ (sense), 5′-GGAGAGA GATGAGAAAGAAGC-3′ (antisense)) were designed to the 5′ and 3′-untranslated regions (UTR) of hPAT1 (identified by alignment of two hPAT1 sequences (GenBank accession numbers NM_078483 and AF516142)). PCR was performed using Expand High Fidelity Polymerase (Roche) and 10 PCR cycles were carried out for 15 s at 94°C, 30 s at 60°C and 2 min at 72°C. In all, 20 cycles were subsequently performed at 94°C for 15 s, 60°C for 30 s, 72°C for 2 min with cycle elongation of 5 s for each cycle. PCR yielded a product of 1.91 kb, as expected from the relative positions of the primers. The product was then cloned into the pCR2.1-TOPO vector (Invitrogen) and construct fidelity was confirmed by DNA sequencing (Molecular Biology Unit, University of Newcastle upon Tyne, U.K.). Linearised hPAT1 plasmid DNA was used as a template for *in vitro* transcription with mMESSAGE mMACHINE Kit (Ambion).

Functional expression of hPAT1 and mPAT1 in Xenopus laevis *oocytes*

X. laevis oocytes were prepared and uptake experiments performed, as described previously (Anderson *et al*., 2004). Briefly, individual stage V/VI oocytes were injected with 50 nl of water (control), hPAT1 (1 μg μl^{-1}) or mouse PAT1 (mPAT1, 1 μg μl^{-1}) (Boll *et al*., 2002; 2003) cRNA and incubated for 2–3 days in Barth's solution at 18°C. [^{3}H]GABA (5 μCi ml^{-1}; 20–100 μM) uptake was measured in the presence/absence of various compounds at room temperature (22°C) over 40 min (Na^+-free, pH 5.5). The oocytes were lysed in 10% SDS before scintillation counting.

Measurement of substrate-induced currents using the two-electrode voltage-clamp technique

Two-electrode voltage-clamp measurements were performed using *X. laevis* oocytes expressing hPAT1 (clamped at −60 mV), as described previously (Anderson *et al*., 2004). The currents induced following addition of various compounds (10 mM, Na^+-free, pH 5.5, 60 s) to the bathing solution of hPAT1 or water-injected oocytes were determined by subtraction of current measured 20 s before the addition of the compound from that 60 s after addition of the compound. Results are presented both as representative traces and as mean substrate-induced current change (following subtraction of current produced by water-injected control oocytes under identical experimental conditions) and are expressed as a percentage of current evoked by 10 mM GABA.

Statistical analysis

Data are mean ± s.e.m. or s.d., as appropriate. Statistical comparisons of mean were made using one-way analysis of variance (ANOVA) using the Bonferroni multiple comparisons post-test or a Student's *t*-test, as appropriate. Curve fitting was performed using GraphPad Prism version 3.0.

Results

Vigabatrin inhibition of hPAT1-mediated amino acid uptake in Caco-2 cell monolayers

pH-dependent, Na^+-independent, PAT1-mediated [^{3}H]β-alanine, [^{3}H]proline and [^{3}H]glycine (all 20 μM) uptake across the apical membrane of Caco-2 cell monolayers was measured (apical pH 5.5, basolateral pH 7.4, Na^+-free buffers). The presence of either 10 mM GABA or 10 mM vigabatrin at the

apical membrane significantly inhibited the uptake of all three PAT1 substrates (Figure 1) (all $P<0.01$ *versus* control) suggesting that vigabatrin transport is *via* a Na^+-independent pH-dependent uptake mechanism shared with the PAT1 substrates β-alanine, proline and glycine. In contrast, apical uptake of the dipeptide [^{14}C]Gly-Sar *via* the human intestinal proton-coupled di/tripeptide transporter (hPepT1) was unaffected ($P>0.05$ *versus* control) by the presence of either GABA or vigabatrin demonstrating that vigabatrin, like GABA, is not absorbed *via* hPepT1 (data not shown).

In Na^+-free conditions, the transcellular transepithelial apical-to-basal [^{3}H]GABA flux (J_{a-b}) (after subtraction of the paracellular component estimated by [^{14}C]mannitol flux) was significantly increased ($P<0.001$) from 621 ± 7 pmol cm^{-2} (90 min)$^{-1}$ ($n=11$) to 1048 ± 35 pmol cm^{-2} (90 min)$^{-1}$ ($n=11$) when apical pH was decreased from pH 7.4 to pH 5.5. This pH-dependent [^{3}H]GABA J_{a-b} is equivalent to the net flux of GABA (J_{net}), as determined previously (Thwaites *et al*., 2000). The pH-dependent [^{3}H]GABA J_{a-b} is reduced (down to levels measured in the absence of a transepithelial pH gradient) by 10 mM unlabelled GABA (486 ± 9 pmol cm^{-2} (90 min)$^{-1}$ ($n=11$)), vigabatrin (499 ± 7 pmol cm^{-2} (90 min)$^{-1}$ ($n=12$)), isonipecotic acid (577 ± 9 pmol cm^{-2} (90 min)$^{-1}$ ($n=11$)) and nipecotic acid (606 ± 9 pmol cm^{-2} (90 min)$^{-1}$ ($n=11$)) (all $P<0.001$ *versus* control, pH 5.5 alone).

To determine whether other GABA derivatives are also likely to be substrates for hPAT1, [^{3}H]GABA (100 μM) uptake across the apical membrane of Caco-2 cell monolayers was measured in the presence of vigabatrin, TACA, guvacine or gabapentin (all 10 mM). [^{3}H]GABA uptake was significantly reduced by vigabatrin, TACA and guvacine (all $P<0.01$ *versus* control) suggesting that these GABA analogues are substrates for hPAT1 (Figure 2). In contrast, the AED gabapentin had no inhibitory effect on [^{3}H]GABA uptake ($P>0.05$ *versus* control). In a separate series of experiments, [^{3}H]GABA uptake (Na^+-free, pH 5.5, at a lower substrate concentration of 20 μM GABA) across the brush-border membrane over 5 min was reduced from 104 ± 4 pmol cm^{-2} ($n=18$) to 27 ± 2 pmol cm^{-2} ($n=18$), 42 ± 2 pmol cm^{-2} ($n=18$) and 39 ± 1 pmol cm^{-2} ($n=5$) in the presence of (all 10 mM) cold GABA, vigabatrin and 4-amino-5-hexynoic acid (AHA or γ-acetylenic GABA), respectively, suggesting that AHA is also a hPAT1 substrate.

Figure 3 demonstrates that vigabatrin (IC_{50} 2.9 ± 1.2 mM) and TACA (IC_{50} 3.6 ± 1.3 mM) both inhibit [^{3}H]GABA uptake across the apical membrane of Caco-2 cell monolayers in a concentration-dependent manner with a similar affinity to that measured using unlabelled GABA as the competitor (IC_{50} 2.4 ± 1.2 mM).

pH_i measurements

Competition experiments alone cannot determine whether a compound is simply an inhibitor of a transporter or a transported substrate. H^+/vigabatrin cotransport was confirmed by measurement of substrate-coupled H^+-flow across

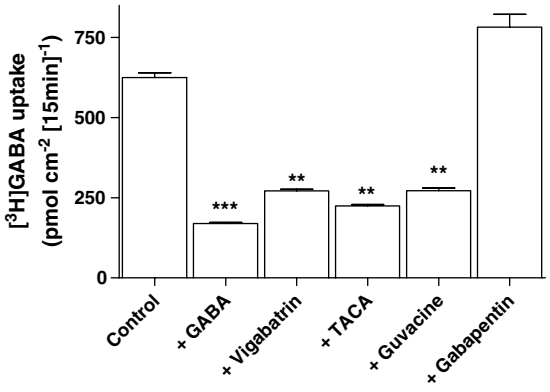

Figure 2 The effects of GABA-related compounds on [^{3}H]GABA uptake across the apical membrane of Caco-2 cell monolayers. Apical [^{3}H]GABA (100 μM) uptake (15 min, apical pH 5.5, basolateral pH 7.4, Na^+-free solutions) in Caco-2 cell monolayers was measured in the presence or absence (control) of unlabelled GABA, vigabatrin, TACA, guvacine or gabapentin (all 10 mM). Data are mean$\pm$s.e.m ($n=6$–12): ***$P<0.001$ or **$P<0.01$ both *versus* control.

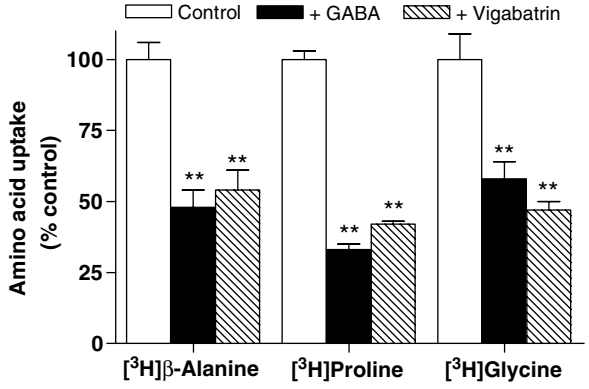

Figure 1 The effects of GABA and vigabatrin on amino acid uptake across the apical membrane of Caco-2 cell monolayers. Apical uptake of [^{3}H]β-alanine, [^{3}H]proline or [^{3}H]glycine (all 20 μM) (5 min, apical pH 5.5, basolateral pH 7.4, Na^+-free solutions) in Caco-2 cell monolayers was measured in the absence (open columns) or presence of unlabelled GABA (filled columns) or vigabatrin (hatched columns) (both 10 mM). Data are mean$\pm$s.e.m. ($n=11$): **$P<0.01$ *versus* control.

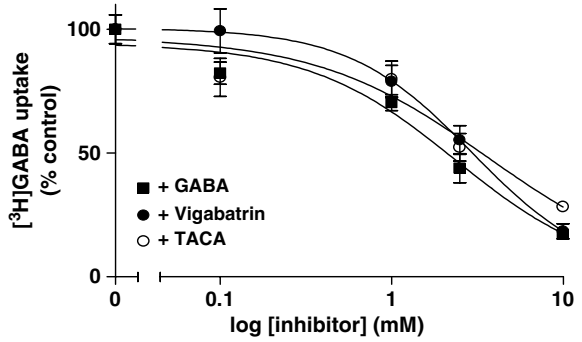

Figure 3 Concentration-dependent inhibition of [^{3}H]GABA uptake across the apical membrane of Caco-2 cells by GABA and related compounds. Apical [^{3}H]GABA (20 μM) uptake (15 min, apical pH 5.5, basolateral pH 7.4, Na^+-free solutions) in Caco-2 cell monolayers was measured in the absence and presence (all 0–10 mM) of unlabelled GABA (filled squares), vigabatrin (filled circles) or TACA (open circles). Data are the mean$\pm$s.e.m. ($n=17$–35). Non carrier-mediated uptake, measured in the presence of excess β-alanine (30 mM), was subtracted from total uptake to determine carrier-mediated uptake.

the apical membrane of BCECF-loaded Caco-2 cell monolayers (Figure 4). When the apical superfusate is changed from a pH 7.4 Na^+-containing solution to a pH 5.5 Na^+-free solution, it is clear that there is a marked intracellular acidification (Figure 4). Once the rate of this acidification due to the presence of apical pH 5.5 alone slowed ($\Delta pH_i\,min^{-1}$ 0.012 ± 0.004, $n=8$), GABA (10 mM) was added to the apical superfusate, which led to an acceleration ($P<0.001$ *versus* pH 5.5 alone) in the rate of acidification ($\Delta pH_i\,min^{-1}$ 0.073 ± 0.007, $n=8$) (Figure 4a). Similarly, when vigabatrin (10 mM) was added to the apical superfusate, the rate of acidification increased ($P<0.001$) from $\Delta pH_i\,min^{-1}$

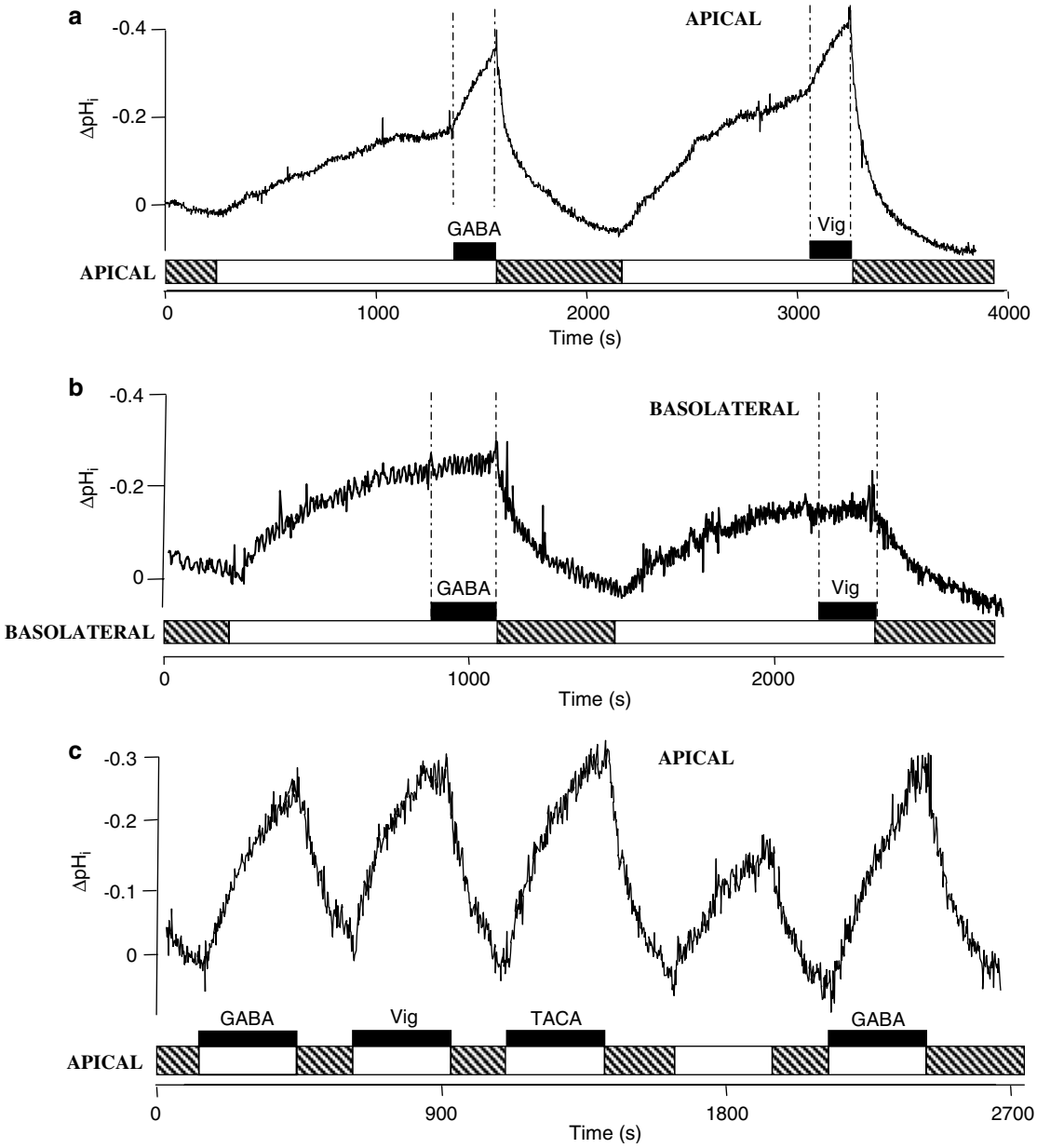

Figure 4 Intracellular pH measurements in BCECF-loaded Caco-2 cell monolayers. (a) The apical surface of the BCECF-loaded Caco-2 cell monolayers were superfused with a Na^+-containing pH 7.4 solution (hatched bar). At the indicated point on the diagram the solution was replaced with Na^+-free pH 5.5 solution (open bar) and subsequently either GABA or vigabatrin (both 10 mM; filled bars) were added to the apical superfusate for 200 s. Replacement of the apical superfusate with a pH 7.4 Na^+-containing buffer (hatched bar) allows pH_i to recover back towards baseline. The basolateral superfusate was pH 7.4 (Na^+-free) throughout. A single representative trace of eight. (b) The basolateral surface of the BCECF-loaded Caco-2 cell monolayers were superfused with a Na^+-containing pH 7.4 solution (hatched bar). At the indicated point on the diagram the solution was replaced with Na^+-free pH 5.5 solution (open bar) and subsequently either GABA or vigabatrin (both 10 mM; filled bars) were added to the basolateral superfusate for 200 s. Replacement of the basolateral superfusate with a pH 7.4 Na^+-containing buffer (hatched bar) allows pH_i to recover back towards baseline. The apical superfusate was pH 7.4 (Na^+-free) throughout. A single representative trace of seven. (c) The effects on pH_i of sequential addition of pH 5.5 (Na^+-free) alone (open bar) or GABA, vigabatrin and TACA (all 10 mM; filled bars) in a Na^+-free pH 5.5 buffer. Basolateral pH 7.4, Na^+-free under all conditions. Where indicated on the figure a Na^+-containing pH 7.4 solution was superfused across the apical surface to allow the cells to recover (hatched bars). A single trace representative of six.

0.015 ± 0.002 ($n=8$) to $\Delta pH_i\,min^{-1}$ 0.055 ± 0.004 ($n=8$) (Figure 4a). In contrast when the manoeuvre was repeated at the basolateral surface of the BCECF-loaded Caco-2 cell monolayers the rate of acidification was not increased when either GABA ($\Delta pH_i\,min^{-1}$ 0.018 ± 0.010 ($n=7$) in pH 5.5 alone and $\Delta pH_i\,min^{-1}$ 0.013 ± 0.004 ($n=7$) after addition of GABA) or vigabatrin ($\Delta pH_i\,min^{-1}$ 0.010 ± 0.004 ($n=7$) in pH 5.5 alone and $\Delta pH_i\,min^{-1}$ 0.011 ± 0.003 ($n=7$) after addition of vigabatrin) was added to the basolateral superfusate (Figure 4b, both $P>0.05$), demonstrating that the H^+/GABA or vigabatrin cotransport mechanism, like hPAT1, is confined to the apical surface of these intestinal enterocytes. As shown in Figure 4c, the initial rate of acidification across the apical membrane of BCECF-loaded Caco-2 cells is similar following apical exposure to GABA, vigabatrin or TACA (all 10 mM, pH 5.5, Na^+-free solutions). The rate of intracellular acidification observed after addition of GABA to the apical superfusate is $198.7 \pm 7.5\%$ ($n=7$) of that observed after superfusion of the pH 5.5 buffer alone; similar observations are made with vigabatrin ($180.6 \pm 16.8\%$ ($n=6$)) and TACA ($171.4 \pm 11.0\%$ ($n=6$)) (all $P<0.01$ *versus* pH 5.5 alone). Thus the ability of vigabatrin and other GABA-derivatives to induce ΔpH_i is matched by their ability to inhibit [^{3}H]GABA uptake, suggesting that they undergo H^+/substrate cotransport *via* hPAT1.

PAT1-mediated amino acid uptake in PAT1-expressing oocytes

In order to confirm that hPAT1 is the endogenous transporter responsible for the uptake of vigabatrin in Caco-2 cell monolayers measurements were made using PAT1-expressing oocytes. To enable direct comparison, transport was measured under identical experimental conditions to those used in the Caco-2 cell studies (extracellular pH 5.5, Na^+-free incubation buffers). Under control conditions, [^{3}H]GABA uptake (20 μM) into hPAT1-expressing oocytes is seven-fold greater than that in water-injected oocytes ($P<0.001$). Excess GABA, vigabatrin, TACA and guvacine all significantly inhibited [^{3}H]GABA uptake ($P<0.01$), whereas gabapentin had no effect ($P>0.05$) (Figure 5a). In separate experiments AHA, GABA and vigabatrin (all 10 mM) all reduced (all $P<0.001$ *versus* control) hPAT1-mediated [^{3}H]GABA uptake from 15.6 ± 1.9 nmol oocyte^{-1} (40 min)$^{-1}$ ($n=18$) to 1.4 ± 0.3 nmol oocyte^{-1} (40 min)$^{-1}$ ($n=18$), 2.3 ± 0.2 nmol oocyte^{-1} (40 min)$^{-1}$ ($n=19$) and 2.0 ± 0.3 nmol oocyte^{-1} (40 min)$^{-1}$ ($n=20$), respectively. Similar observations are made using mPAT1-expressing oocytes where GABA and vigabatrin (both 10 mM) markedly inhibited uptake of the PAT1 substrates [^{3}H]GABA, [^{3}H]β-alanine, [^{3}H]glycine and [^{3}H]proline ($P<0.01$ *versus* control) (Figure 5b).

The ability of vigabatrin and other GABA-related compounds to induce inward current (due to electrogenic H^+/substrate cotransport) in hPAT1-expressing oocytes was determined. Figure 6 demonstrates that extracellular exposure of hPAT1-expressing oocytes to GABA, vigabatrin, TACA and guvacine is associated with a large inward current not observed in water-injected oocytes. The current responses to vigabatrin ($75.7 \pm 4.0\%$ of the GABA response, mean $\pm$ s.d., $n=3$), TACA ($73.2 \pm 2.6\%$, $n=3$) and guvacine ($87.6 \pm 3.8\%$, $n=3$) are similar to that induced by GABA alone (all $P>0.05$ *versus* GABA, all $P<0.001$ *versus* water). Thus, the ability of these GABA analogues to inhibit radiolabelled [^{3}H]GABA uptake is similar to their ability to induce inward current, demonstrating that GABA, vigabatrin, TACA and guvacine are all substrates for transport *via* hPAT1.

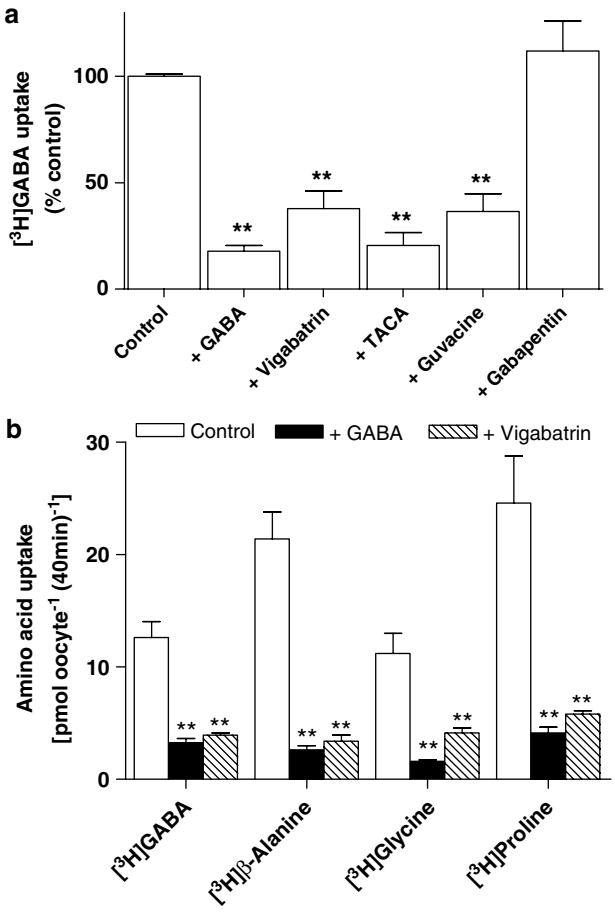

Figure 5 The effects of GABA and related compounds on amino acid uptake in PAT1-expressing oocytes. (a) [^{3}H]GABA (20 μM) uptake (pH 5.5, Na^+-free) *via* hPAT1 was measured in the absence (control) or presence of unlabelled GABA, vigabatrin, TACA, guvacine or gabapentin (all 10 mM). Data are expressed as a percentage control ([^{3}H]GABA uptake in the absence of unlabelled compounds) after subtraction of uptake in water-injected oocytes under identical experimental conditions. Data are mean $\pm$ s.e.m. ($n=18$–20): $^{**}P<0.01$ *versus* control. (b) [^{3}H]GABA, [^{3}H]β-alanine, [^{3}H]glycine, [^{3}H]proline (all 20 μM) uptake (pH 5.5, Na^+-free) *via* mPAT1 was measured in the absence (open columns) or presence of GABA (filled columns) or vigabatrin (hatched columns) (both 10 mM). Data are expressed as mean $\pm$ s.e.m. ($n=10$) following subtraction of uptake into water-injected oocytes under identical experimental conditions: $^{**}P<0.01$ *versus* control.

Discussion

The H^+-coupled amino-acid transporter (system PAT) at the brush-border membrane of mammalian intestinal epithelia transports a broad range of amino acids and orally delivered therapeutic compounds (Thwaites *et al*., 1993a, b; 1994; 1995a, b, c; Thwaites & Stevens, 1999; Thwaites *et al*., 2000; Boll *et al*., 2003; Metzner *et al*., 2004). cDNAs representing system PAT have been isolated from rat brain (LYAAT1) (Sagne *et al*., 2001), mouse (mPAT1) (Boll *et al*., 2002) and human

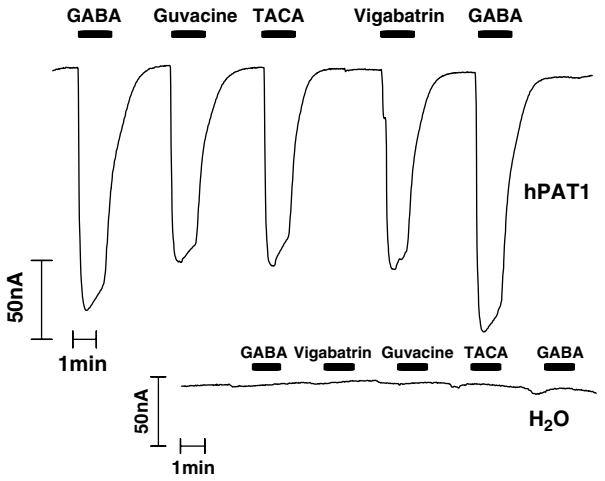

Figure 6 Current changes induced by GABA and related compounds in hPAT1-expressing oocytes. Single representative trace (of three) showing current changes in hPAT1 and water-injected oocytes (insert) following exposure (as indicated by bars) to GABA, vigabatrin, TACA or guvacine (all 10 mM, pH 5.5, Na^+-free).

intestine (hPAT1) (Chen *et al*., 2003). Studies using Caco-2 cell monolayers and PAT1-expressing *X. laevis* oocytes have used a combination of radiotracer flux studies and measurements of substrate-coupled H^+-influx (using BCECF-loaded Caco-2 cell monolayers) or inward current (using the two-electrode voltage clamp technique in oocytes) to determine the structural requirements that allow interaction of a substrate with PAT1 (Thwaites *et al*., 1995c; Boll *et al*., 2003). By analysis of the transport and relative affinity of a large array of test compounds, the two major conformational limitations of the PAT1 active centre appear to be the size of the amino acid side chain and the length of the backbone (Boll *et al*., 2003). Such insight into the restrictions in the PAT1 binding site enables potential PAT1 substrates to be identified and highlights the potential of PAT1 as a route for oral delivery of therapeutic compounds.

Previous investigations have demonstrated that GABA and GABA analogues (nipecotic acid, isonipecotic acid, β-aminobutyric acid and 3-amino-1-propanesulphonic acid) can undergo rapid transport across the apical membrane of intestinal epithelial (Caco-2) cell monolayers *via* PAT1 (Thwaites *et al*., 2000). Therefore, the aim of this study was to determine if the orally delivered GABA analogues vigabatrin and gabapentin, used in the treatment of partial and focal epilepsy, and the nonclinically administered GABA derivatives TACA, guvacine and AHA are substrates for the PAT1 carrier.

We have shown through radiolabelled uptake experiments (Figure 1) that the GABA analogue vigabatrin inhibits uptake of known PAT1 substrates across the apical membrane of Caco-2 cell monolayers. Additional competition experiments verify that vigabatrin transport is concentration-dependent with a similar affinity to that obtained with other PAT1 substrates (Figures 2 and 3). The exclusive apical localisation of vigabatrin-induced H^+-influx further supports the hypothesis that vigabatrin uptake is mediated *via* hPAT1 (Figure 4). In order to confirm that hPAT1 is the endogenous transporter responsible for the uptake of vigabatrin in Caco-2 cell monolayers, vigabatrin transport was investigated in oocytes injected with hPAT1 or mPAT1 cRNA. Competition experiments confirmed that vigabatrin inhibits uptake of GABA and other known PAT1 substrates in PAT1-expressing oocytes (Figure 5). Two-electrode voltage-clamp measurements, using hPAT1-expressing oocytes, confirm that vigabatrin transport is associated with an inward current (H^+/vigabatrin cotransport) not observed in water-injected oocytes (Figure 6). In agreement with the proposed model of the PAT1 binding site, the linear structures of vigabatrin, TACA, and AHA and guvacine, where the α-carbon and imino group are incorporated into the heterocyclic structure, enables interaction with the PAT1 binding pocket. In contrast, the large side chain on the β-carbon of gabapentin predictably excludes this compound from PAT1 binding. Therefore, this study supports the hypothesis that one of the major restrictions for PAT1-binding is the size of the amino-acid side chain.

Although direct measurement of transepithelial transport of vigabatrin is necessary to verify that vigabatrin exits the basolateral membrane of these intestinal epithelial cells, previous investigations with other PAT1 substrates, namely β-alanine, L-alanine, α-methylaminoisobutyric acid, proline, GABA and glycine (Thwaites *et al*., 1993a, b; 1994; 1995a, b, c; 2000), when considered alongside the high oral bioavailability of vigabatrin, suggest that transepithelial transport occurs. Potential efflux systems localised to the basolateral membrane of the small intestine include system L (CD98/LAT2, SLC3A2/SLC7A8) which exchanges neutral amino acids (Rossier *et al*., 1999; Bauch *et al*., 2003), system y^+L (CD98/y^+LAT1, SLC3A2/SLC7A7), which exchanges neutral amino acids for intracellular cationic amino acids in the presence of sodium (Kanai *et al*., 2000; Bauch *et al*., 2003), or system asc (CD98/asc1, SLC3A2/SLC7A10), which is a Na^+-independent, high-affinity neutral amino-acid exchanger (Wagner *et al*., 2001). However, the specific carrier system(s) involved in vigabatrin transport across the basolateral membrane has yet to be determined.

Gabapentin absorption by rat and rabbit brush-border membrane vesicles (BBMV) is sodium-independent and greater in duodenal and ileal BBMV compared with jejunal BBMV (Piyapolrungroj *et al*., 2001). It has been suggested that the intestinal uptake of gabapentin across the brush-border membrane may be mediated by system $b^{0,+}$ (rBAT/$b^{0,+}$AT, SLC3A1/SLC7A9), which transports neutral and dibasic amino acids in a sodium-independent manner (Wagner *et al*., 2001). In a rat intestinal everted ring preparation, gabapentin uptake shared an inhibition profile with L-phenylalanine, suggesting that system L may play a role in basolateral transport of gabapentin (Stewart *et al*., 1993). However, further investigation into the route of gabapentin intestinal absorption is required.

Any orally delivered compound used to treat a CNS disorder (e.g. epilepsy) must cross several distinct barriers before reaching the target site of action. These barriers include the small intestinal epithelium (both apical and basolateral membranes), the blood–brain barrier (BBB) and, in the case of a drug with an intracellular site of action, the neuronal and/or glial cell membrane. The various transport mechanisms responsible for movement of vigabatrin across these membranes are not known. However, vigabatrin does inhibit carnitine uptake in the human placenta probably *via* the organic cation transporter OCTN2 (SLC22A5) (Wu *et al*., 2004). OCTN2 is expressed at the BBB (Berezowski *et al*., 2004). Vigabatrin also inhibits GABA transport *via* the Na^+-

dependent GABA transporter GAT1 (SLC6A1) (Eckstein-Ludwig *et al*., 1999) which may represent a route for vigabatrin influx into both neurones and glial cells. The role of PAT1 in vigabatrin transport in neural tissues is not clear as PAT1 is found mainly in lysosomes in neurones (Sagne *et al*., 2001; Agulhon *et al*., 2003) although functional PAT1-like expression has also been detected at the plasma membrane of cultured hippocampal neurones (Wreden *et al*., 2003).

The present study demonstrates that vigabatrin is a substrate for the H^+-coupled amino-acid transporter hPAT1, which is expressed at the brush-border membrane of the human small intestinal epithelium. The high capacity of hPAT1 partially explains the high oral bioavailability of vigabatrin (80–90%) and emphasises the therapeutic significance of hPAT1 as a route for the oral absorption of clinically administered GABA-related compounds.

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