Molecular cloning and functional characterization of a GABA/betaine transporter from human kidney

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Received 5 May 1995; revised version received 5 September 1995

Abstract The human homologue of the canine GABA/betaine transporter (BGT-1) was isolated from a kidney inner medulla cDNA library. The coding sequence predicts a 614 amino acids protein with the typical features of neurotransmitter transporter family. The gene maps to chromosome 12p13 and, in addition to kidney, is also expressed in brain, liver, heart, skeletal muscle, and placenta. Functional studies reveal a K_m = 20 μM for GABA transport and a coupling to Na^+ and Cl^- with a stoichiometry 3 Na^+:2 Cl^-:1 GABA. At 500 μM the GABA transport was inhibited by various compounds with the following potency order: quinidine > verapamil > phloretin > betaine.

Key words: GABA; Betaine; Kidney; Transporter; Cell volume; Chromosomal localization

1. Introduction

Volume regulation is accomplished by several cell types through active variations in the concentration of intracellular solutes. In particular, epithelial cells from the renal inner medulla balance the extracellular hypertonicity by keeping a high intracellular content of non-perturbing osmolytes like betaine, glycerophosphorylcholine, myo-inositol, sorbitol, and taurine [1]. Intracellular accumulation of some of these compounds up to millimolar concentrations is performed by membrane transporters which use the favourable driving force for Na^+ and Cl^-. Notably, molecular cloning of taurine and betaine transporters from kidney has revealed a strong sequence homology with a large superfamily of proteins involved in the uptake of neurotransmitters [2]. These transporters are responsible for the termination of synaptic transmission by lowering the level of the neurotransmitter in the synaptic cleft. Interestingly, the canine betaine transporter (BGT-1) is also able to transport the inhibitory neurotransmitter GABA with even higher affinity [3]. This characteristic would suggest that GABA/betaine transporters have a dual function in osmoregulation and synaptic transmission.

Given the importance of these mechanisms to kidney physiology and the possible involvement in human diseases, we undertook the identification of the human gene homologue of BGT-1. In the present work we report the molecular cloning, functional characterization, tissue distribution, and chromosomal localization of a cDNA identified by screening a human kidney cDNA library with a BGT-1 probe.

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2. Materials and methods

2.1 cDNA cloning and sequencing

A human cDNA library from the inner medulla of the kidney was constructed in the λgt22 vector (BRL, Life Technologies) using *Not*I and *Sal*I cloning sites. The library was screened with a PCR product obtained from human cDNA. To obtain this probe, different primer pairs were designed based on the dog BGT-1 sequence and tested. A 1.5 kb amplification product was obtained with the following primers: forward, 5′-TTTCACAGCCACGTTTCC-3′, and reverse, 5′-CGTTCAGTTGTCACTTCC-3′, corresponding respectively to positions 720 and 2269 in the BGT-1 sequence [3]. PCR conditions were: 35 cycles, each of them composed by 1 min at 94°C, 1 min at 44°C, and 1 min 30 s at 72°C, with a denaturing step at the beginning (3 min, 94°C) and an elongation step at the end (5 min, 72°C). This PCR product was cloned in a plasmid vector (TA cloning kit, Invitrogen) and sequenced using the Sequenase kit (U.S. Biochemicals). The sequence revealed strong homology with the canine GABA/betaine transporter. To screen the library, the amplification product was radiolabeled by random priming with [α-^{32}P]dCTP (Amersham). Phage DNA from positive clones was extracted with a commercially available kit (Qiagen). The DNA was *Not*I/*Sal*I digested and Southern blots were performed with the PCR BGT-1 probe to analyze inserts. Largest positive cDNAs identified in this way were cloned in the pSPORT1 vector (BRL) previously cut with *Not*I and *Sal*I. Recombinant transformations were verified by digestion and PCR analysis performed with either SP6/T7 primers and the specific primers reported above. A clone termed B18 showed an insert of about 3.4 kb whose nucleotide sequence was determined on both strands.

2.2. Northern-blot analysis

The cloned amplification product used for the screening of the library was radiolabeled and hybridized to a Northern-blot prepared with 2 μg of poly(A)$^+$ RNA from multiple human tissues (human MTN blot, Clontech). Hybridization was carried out according to manufacturer's instruction. Filter was washed at high stringency with 2 × SSC, 0.05% SDS; then 2 × SSC, 0.1% SDS; 1 × SSC, 0.1% SDS; and finally 0.5 × SSC, 0.1% SDS.

2.3. Chromosomal localization

Chromosomal localization was obtained by fluorescent in situ hybridization on metaphase chromosomes prepared from a human lymphoblastoid cell line by routine methods. Phage λgt22 DNA containing the B18 insert was labeled by nick translation (Boehringer) using biotin-16-dUTP. Signals were amplified twice and the slides were stained with propidium iodide and DAPI.

2.4. cDNA transient expression

To perform functional studies the B18 cDNA clone, which represents the putative full-lenght coding sequence of the transporter, was excised from the pSPORT1 vector and cloned in the eukaryotic expression vector pcDNAI (Invitrogen) as a *Pst*I/*Not*I fragment. Transient expression was achieved by electroporation of human tracheal 9HTEo- cells [4]. Briefly, the cells, detached by trypsinization, were washed in PBS, centrifuged, and resuspended in an ice-cold solution whose composition mimicked the intracellular environment [5]. Aliquots of $6 \cdot 10^6$ cells in 0.7 ml of this solution were mixed with 20 μg of the recombinant pcDNAI plasmid or with the same amount of the plasmid without the insert as negative control. Cell/DNA suspensions were placed on ice for 10 minutes and then electroporated with a Gene Pulser (Bio-Rad), using

SSDI 0014-5793(95)01052-1

a voltage pulse of 1.2 kV and a capacitance of 25 μF. After 10 minutes on ice, cells were diluted in culture medium (DMEM/F12 plus 10% fetal calf serum) and plated in 24-well plates at a density of $3 \cdot 10^5$ cells per well. Uptake experiments were carried out after 60 hours from electroporation.

2.5. GABA uptake

Culture medium was removed and cells were washed with a solution containing (in mM): 130 NaCl, 2 KCl, 1 KH_2PO_4, 2 $CaCl_2$, 2 $MgCl_2$, 10 Na-HEPES, 10 glucose, 20 mannitol (pH 7.3). Cells were then incubated with 1 ml of the same saline solution containing in addition 0.5 μCi of [^{3}H]GABA (specific activity 90 Ci/mmol, Amersham) and the appropriate amount of non radioactive GABA to obtain the desired concentration. Uptake was stopped by washing the cells three times with 1 ml of ice cold solution without GABA. Cells were then solubilized overnight in 0.5 ml of 0.25 M NaOH and 20 μl of cell lysate were used to determine the protein content of each well with a colorimetric assay (Bio-Rad). The remaining lysate was used to detect the radioactivity by liquid scintillation counting. Values of radioactivity incorporated were converted in nanomoles of GABA per mg of protein. To evaluate the dependence of uptake on medium composition, Na^+ or Cl^- were partially or totally replaced by equimolar amounts of *N*-methyl-D-glucamine or aspartate, respectively.

3. Results

Sequencing of B18, the largest clone obtained by screening of the human kidney cDNA library, showed an insert of 3433 bp containing an open reading frame of 1842 bp which encodes a protein of 614 amino acids. This sequence has been deposited in GenBank with the accession number U27699. Comparison of the coding sequence in our clone with that of a cDNA recently isolated from human brain [6] revealed only minor differences thus indicating that they arise from the same gene. In particular, three of these involve amino acid changes: $R_{10} \rightarrow Y_{10}$, $H_{571}V_{572} \rightarrow Q_{571}L_{572}$. The reason for these differences is not clear, but cannot be attributed to a sequence artifact. None of these substitutions seems to alter a known functional site. In addition, the B18 clone extends 526 bp at the 5′-terminus and 990 bp at the 3′-terminus with respect to the sequence found by Borden et al. [6]. Browsing of sequence databases revealed a strong homology with the family of neurotransmitter transporter genes [2]. As shown in Fig. 1, there is a high amino acid sequence identity with the dog BGT-1 [3], the mouse GABA/betaine transporter GAT-2 [7], and GABA transporters GAT-3 and GAT-4 [8]. Hydropathy analysis according to the Kyte–Doolittle plot confirmed the typical features of neurotransmitter transporters, i.e. 12 transmembrane stretches with a large extracellular loop between the third and the fourth transmembrane domains. A search for known consensus sequences revealed three putative protein kinase C phosphorylation sites in the cytosolic portion and two *N*-glycosilation sites in the large extracellular loop (Fig. 1).

Northern blot analysis showed an interesting pattern of tissue distribution (Fig. 2). Two bands of 4.1 and 3.4 kb were observed in kidney, brain (where the former one was faint but clearly detectable with a longer exposition), and weakly in placenta. The 3.4 kb band was also expressed in liver whereas the 4.1 kb band was present in heart and skeletal muscle. The last

Fig. 1. Deduced amino acid sequence of the human GABA/betaine transporter (hBGT-1) compared with the sequences of the corresponding canine transporter (dBGT-1), and murine GABA transporters (GAT-2, GAT-3, GAT-4). Proposed transmembrane domains are underlined and numbered. Asterisks indicate amino acids identical in the five sequences. Triangles and crosses mark putative sites of N-linked glygosilation and PKC-mediated phosphorylation, respectively.

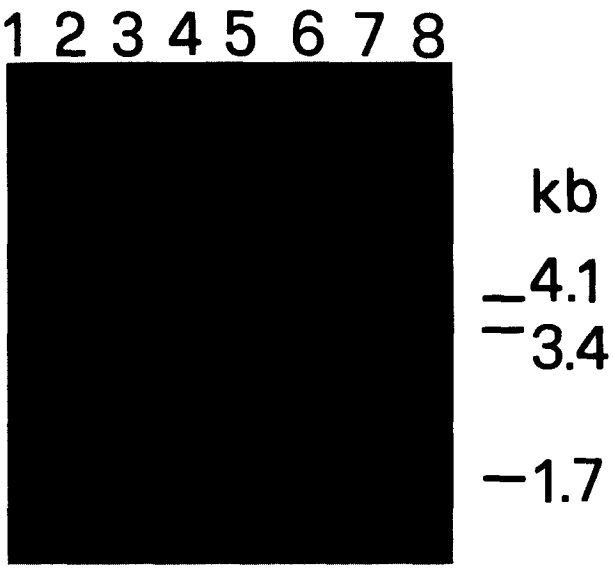

Fig. 2. Tissue expression of hBGT-1 gene determined by Northern blot analysis. Tissues are: 1, pancreas; 2, kidney; 3, skeletal muscle; 4, liver; 5, lung; 6, placenta; 7, brain; 8, heart.

tissue also showed a 1.7 kb signal. No expression was instead apparent in lungs and pancreas.

Chromosomal localization was carried out using the fluorescent in situ hybridization technique. Hybridization signals were present in at least 80 human chromosomes in position 12p13 and were concordant in all the metaphases analysed.

Expression of the B18 clone in 9HTEo- cells caused a significantly higher GABA uptake compared with the same cells electroporated with the vector alone. Preliminary experiments showed that the rate of GABA uptake was constant up to hour of incubation in both control and B18-expressing cells (Fig. 3). According to this, the uptake at 30 minutes was used to calculate the kinetic parameters of GABA transport. Mock transfected cells showed an uptake activity which displayed no saturation in the range 1–250 μM of GABA (Fig. 4A). This probably reflects the presence of an endogenous transporter

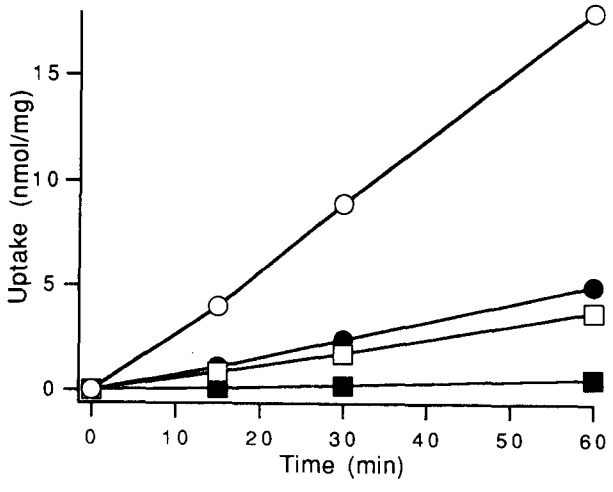

Fig. 3. Time dependence of GABA uptake in 9HTEo- cells transfected with the B18 cDNA clone (open symbols) or with the vector alone (filled symbols). GABA concentration was 100 μM (circles) or 10 μM (squares). Data are from a representative experiment.

which is able to transport GABA with a very low affinity. On the contrary, cells transfected with the B18 clone displayed a markedly higher uptake rate and saturation at high GABA concentrations. This was more evident after subtraction of the GABA transport activity measured in control cells from that measured in cDNA-transfected cells. Subtracted data showed saturation kinetics which could be well fitted with the Michaelis–Menten equation (Fig. 4A). Eadie–Hofstee analysis of 9 experiments performed in triplicate gave a K_m = 20.4 ± 1.6 μM and a V_{max} = 4.128 ± 0.787 nmol/ mg (mean ± S.E.M.; see Fig. 4B).

Coupling of GABA uptake to Na^+ and Cl^- was also investigated. Fig. 5 shows a representative experiment in which the endogenous component was subtracted from the total uptake in cDNA-transfected cells. As shown in Fig. 5, the relationship between GABA uptake rate and Na^+ or Cl^- concentration was sigmoidal suggesting the movement of more than one ion for each molecule of GABA transported. To calculate the number n of ions involved, experimental data were fitted with the Hill-type equation:

$$v = V_{max}[X]^n/(K_{0.5}{}^n + [X]^n)$$

where v is the uptake rate, $K_{0.5}$ is the ion concentration allowing half-maximal activation, X is the ion coupled to GABA transport, and V_{max} is the maximal uptake rate. The best values for n obtained from three separate experiments perfomed in triplicate were 3.2 ± 0.1 and 1.7 ± 0.2 (mean ± S.E.M.) for Na^+ and Cl^-, respectively.

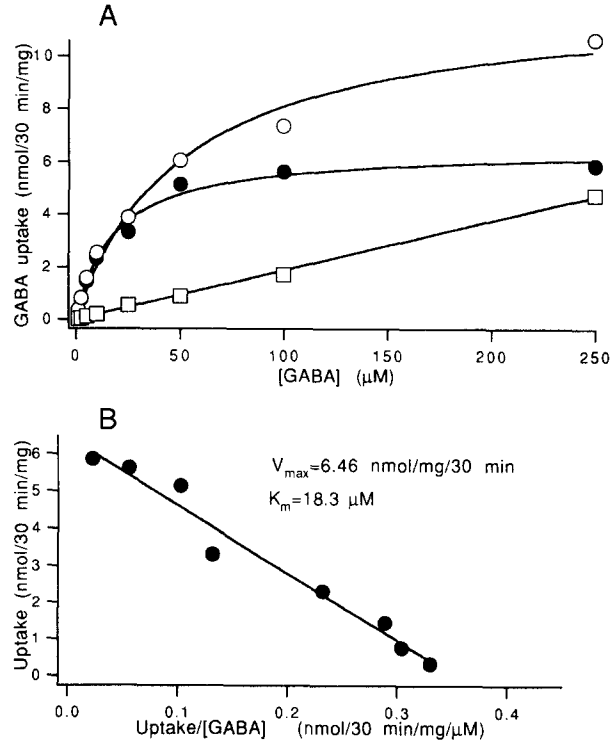

Fig. 4. Kinetics of GABA uptake. Data are from a representative experiment performed in triplicate. Standard errors are smaller than 5% of the corresponding mean value. (A) GABA uptake rate is plotted against the GABA concentration. Symbols are: open circles, hBGT-1 cDNA-transfected cells; open squares, mock-transfected cells; filled circles, difference between former and latter data. (B) Eadie–Hofstee transformation of subtracted data from the same experiment.

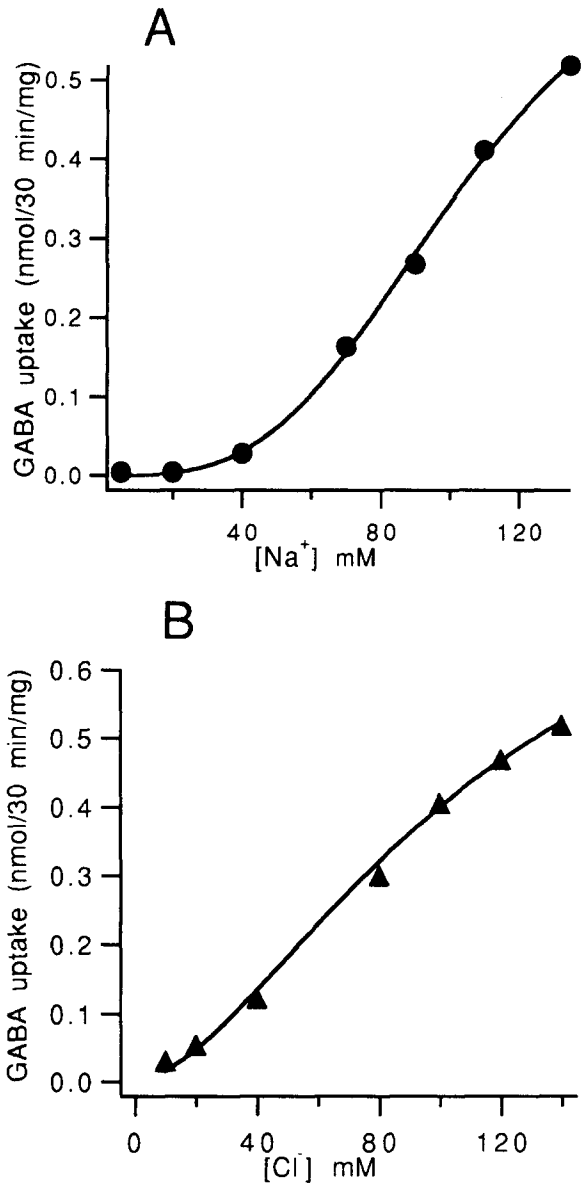

Fig. 5. Dependence of GABA transport on extracellular ions. The net GABA uptake rate induced by the transfection of the transporter cDNA is plotted versus Na^+ (A) and Cl^- (B) concentration. Extracellular GABA concentration was 1 μM. Data are from a representative experiment. Fitting of experimental points with a Hill-type equation gives a n of 3.3 and 1.7 for Na^+ and Cl^-, respectively.

To further characterize the transporter encoded by the B18 clone, different pharmacological agents were tested (Fig. 6). Betaine caused a dose dependent inhibition of GABA uptake with 500 μM being the concentration producing almost 50% of effect. At this same concentration quinidine, phloretin, and verapamil reduced the uptake by 94%, 64% and 69%, respectively. On the contrary, 500 μM taurine did not significantly affect the transporter activity. The protein kinase C activator, phorbol 12-myristate 13-acetate (PMA), was also tested since the amino acid sequence contained putative protein kinase C phosphorylation sites. Preincubation of cells with PMA 1 and 0.1 μM for 10 minutes significantly inhibited GABA uptake by 29% and 13%, respectively.

A more detailed analysis was perfomed for betaine, verapamil, and quinidine to determine the type of inhibition. Fig. 7 shows representative Eadie–Hofstee plots of GABA uptake in the absence and presence of 1 mM betaine, 500 μM verapamil, and 200 μM quinidine. In the presence of betaine, K_m and V_{max} were 62.9 ± 17.1 μM and 3.939 ± 0.927 nmol/mg, respectively (mean ± S.E.M., n = 3). The former value was significantly higher than in control conditions ($P < 0.01$), whereas the latter was not changed thus indicating that betaine acts as a pure competitive inhibitor. On the contrary, verapamil in 5 experiments behaved as a non-competitive inhibitor since it significantly lowered V_{max} to 0.966 ± 0.29 nmol/mg ($P < 0.02$) while leaving unchanged the K_m (20.4 ± 4,7 μM). Quinidine instead showed mixed kinetics because it increased K_m to 49.0 ± 10.4 μM and lowered V_{max} to 2.276 ± 0.499 nmol/mg (n = 3, $P < 0.05$ for both values).

4. Discussion

Na^+/Cl^--dependent monoamines and amino acids transporters constitute a rapidly developing area of interest and are characterized by a high degree of sequence homology [2]. Two subfamilies can be identified within this class of proteins: one is composed by the catecholamine transporters [9–11], whereas the other includes the amino acid-like transporters such as the choline and creatine transporters [12,13], GABA transporters [7,8,14], and the taurine transporter [15] which seems to resemble more closely the putative common ancestor for this subfamily [8]. Interestingly, the physiological role of GABA transporters (GATs) seems to be complicated by the fact that they can carry also other compounds such as β-alanine and taurine (mouse GAT-3 and GAT-4) [8], or betaine (mouse GAT-2) [7]. In particular, the high sequence identity between mouse GAT-2 and dog BGT-1 and the similar pharmacological properties

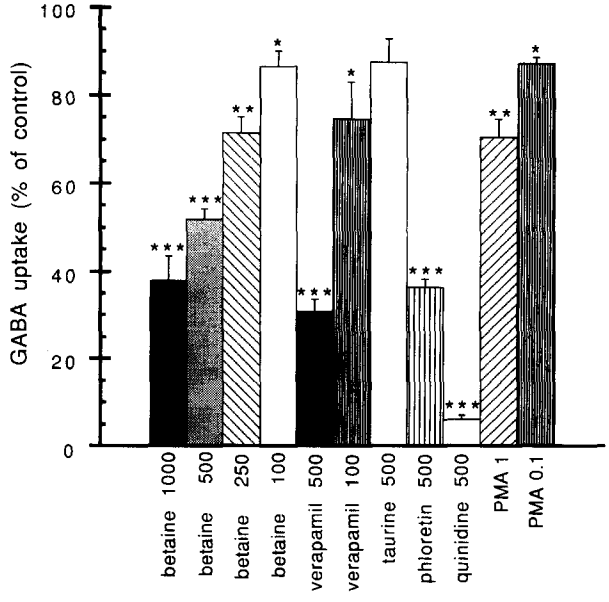

Fig. 6. Residual GABA uptake following application of the indicated compounds (concentrations are given in μM). Data represent the normalized net uptake in cDNA-transfected cells after removal of the endogenous component. Extracellular GABA concentration was 10 μM. Columns represent the mean of three independent experiments. Error bars indicate standard deviation. A significant difference with respect to control is indicated by asterisks: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ (Student's t-test).

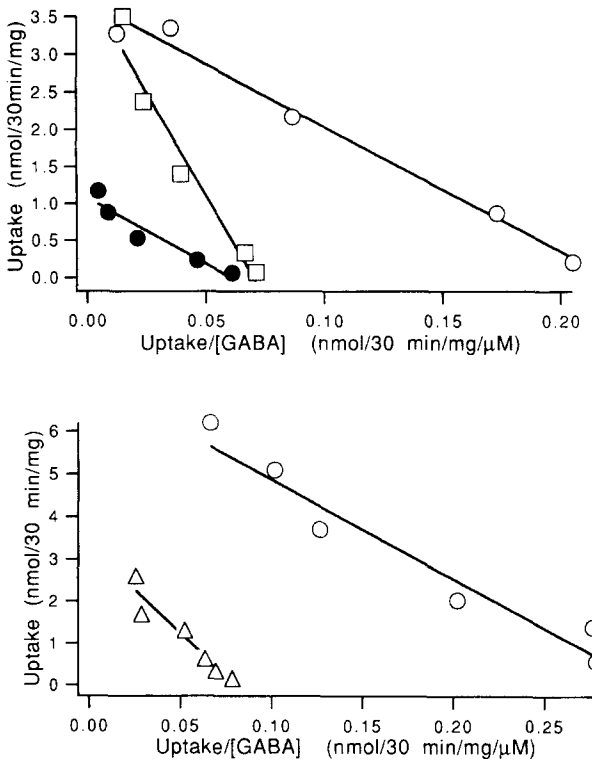

Fig. 7. Kinetics of GABA uptake in the absence (open circles) and presence of 1 mM betaine (open squares), 500 μM verapamil (filled circles), or 200 μM quinidine (open triangles). Data are from 2 representative experiments and are presented as net uptake in cDNA-transfected cells.

suggest that they are the same betaine/GABA transporter, which could therefore be involved either in the control of GABAergic synaptic transmission and in osmoregulation, depending on the site of expression. While preparing this manuscript, Borden et al. have identified the human homologue of BGT-1 and GAT-2 in brain striatum [6]. Our group has independently cloned this gene (hBGT-1) starting from a different tissue, kidney inner medulla. In addition, we have extended the knowledge on hBGT-1 by looking at the pattern of gene expression outside the brain, by determining kinetics parameters of GABA transport and inhibitors, and by mapping the gene.

Our results show that hBGT-1 has a good affinity for GABA, with a K_m of about 20 μM, significantly lower than that of canine BGT-1 [3] and mouse GAT-2 [7], higher than that of GAT-1 and GAT-4 [8,14], and similar to that of mouse GAT-3 [8]. The lack of commercially available radiolabeled betaine has precluded the possibility to study its transport in this work. Nevertheless, the competitive inhibition of GABA uptake by non-radioactive betaine ($IC_{50} \approx 500$ μM) indicates that betaine is also transported but with an affinity at least one order of magnitude lower than that for GABA. Activation of GABA transport as a function of Na^+ and Cl^- concentrations shows cooperativity with a probable stoichiometry of 3 Na^+ and 2 Cl^- for each molecule of GABA transported.

Pharmacological studies showed that hBGT-1 is inhibited by phloretin, and quinidine but not by taurine. Interestingly, and in contrast with data presented by Borden et al. [6], quinidine was the most potent blocker. In addition, we discovered that verapamil, a compound never tested before on neurotransmitter transporters, was also able to inhibit GABA uptake in a non-competitive fashion. It would be interesting to assess in future studies if it is also active on other members of this protein family. The activity of hBGT-1 was also reduced by incubation with the phorbol ester PMA though at relatively high concentrations. Further studies are needed to evaluate the actual involvement of PKC in the inhibition of hBGT-1.

The expression of hBGT-1 is quite widespread, having been found in kidney, brain, liver, heart, skeletal muscle and to a lesser extent in placenta. This pattern resembles that observed in mouse [7], whereas canine BGT-1 was found exclusively in kidney [3]. The physiological meaning of this kind of expression is not clear, and it is made more complex by the presence of at least three transcription products which may arise from alternative splicing. Being betaine plasma levels about two hundred times those of GABA (180 μM vs. less than 1 μM), one should assume that GABA uptake function is restricted to the CNS. Outside the CNS, the major role for hBGT-1 could be the uptake of betaine to contrast a hyperosmotic extracellular medium. Nevertheless, prolonged hypertonic shocks are only experienced by kidney inner medulla during the concentration of urine. Therefore, the possibility of additional functions for this transporter cannot be ruled out and needs further investigation.

Acknowledgements: This work was supported in part from grants from AIRC (Associazione Italiana per la Ricerca sul Cancro) to L.J.V.G. We would like to thank Dr. Olga Zegarra-Moran for help in data analysis and for reading the manuscript, and Giuseppe Santamaria for technical assistance.

References

[1] Bagnasco, S., Balaban, R., Fales, H.M., Yang, Y.-M. and Burg, M.B. (1986) J. Biol. Chem. 26, 5872–5877.
[2] Uhl, G.R. and Hartig, P.R. (1992) Trends Pharmacol. Sci. 13, 421–425.
[3] Yamauchi, A., Uchida, S., Kwon, H.M., Preston, A.S., Robey, R.B., Garcia-Perez, A., Burg, M.B. and Handler, J.S. (1992) J. Biol. Chem. 267, 649–652.
[4] Gruenert, D.C., Basbaum, C.B., Welsh, M.J., Li, M., Finkbeiner, W.E. and Nadel, J.A. (1988) Proc. Natl. Acad. Sci. USA 85, 5951–5955.
[5] Van den Hoff, M.J.B., Moorman, A.F.M. and Lamers, W.H. (1992) Nucleic Acids Res. 20, 2902.
[6] Borden, L.A., Smith, K.E., Gustafson, E.L., Branchek, T.A. and Weinshank, R.L. (1995) J. Neurochem. 64, 977–984.
[7] Lopez-Corcuera, B., Liu, Q.R., Mandiyan, S., Nelson, H. and Nelson, N. (1992) J. Biol. Chem. 267, 17491–17493.
[8] Liu, Q.R., Lopez-Corcuera, B., Mandiyan, S., Nelson, H. and Nelson, N. (1993) J. Biol. Chem. 268, 2106–2112.
[9] Pacholczyk, T., Blakely, R.D. and Amara, S.G. (1991) Nature 350, 350–353.
[10] Shimada, S., Kitayama, S., Lin, C.L., Patel, A., Nanthakumar, E., Gregor, P., Kuhar, M. and Uhl, G. (1991) Science 254, 576–578.
[11] Hoffman, B.J., Mezey, E. and Brownstein, M.G. (1991) Science 254, 579–580.
[12] Mayser, W., Schloss, P. and Betz, H. (1992) FEBS Lett. 305, 31–36.
[13] Nash, S.R., Giros, B., Kingsmore, S.F., Rochelle, J.M., Suter, S.T., Gregor, P., Seldin, M.F. and Caron, M.G. (1994) Receptors and Channels 2, 165–174.
[14] Guastella, J., Nelson, N., Nelson, H., Czyzyk, L., Keynan, S., Miedel, M.C., Davidson, N., Lester, H.A. and Kanner, B.I. (1990) Science 249, 1303–1306.
[15] Uchida, S., Kwon, H.M., Yamauchi, A., Preston, A.S., Marumo, F. and Handler, J.S. (1992) Proc. Natl. Acad. Sci. USA 89, 8230–8234.