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Baclofen (β-*p*-chlorophenyl-γ-aminobutyric acid) transport across rat jejunum

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Intestinal transport of baclofen (β-(*p*-chlorophenyl)-γ-aminobutyric acid) in the rat has been examined in vitro. Influx of baclofen across the brush-border membrane (J_{mc}^{Bacl}) and steady-state accumulation by everted segments of the intestine were measured. J_{mc}^{Bacl} could be accounted for as the sum of a saturable process with a maximum rate of approx. 10–20 nmol cm^{-2} h^{-1} and a $K_{1/2}^{Bacl}$ of approx. 0.3 mM and a diffusive contribution with a permeability of 0.073 cm h^{-1}. J_{mc}^{Bacl} was Na^+- and Cl^--independent. The steady state distribution ratio between the intracellular space of the everted segments and incubation fluid was 1.0 ± 0.1 ($n = 12$). Inhibition tests demonstrated that the Na^+- and Cl^--independent, passive, but saturable fraction of baclofen transport can not be mediated by any of the known amino acid carriers of the rat small intestine. Preliminary results suggests that qualitatively baclofen transport in guinea-pig and rabbit is also by facilitated transport.

Introduction

The spasmolytic drug baclofen (β-(*p*-chlorophenyl)-γ-aminobutyric acid) is a non-α-amino acid. In rat small intestine two transporters have been described for non-α-amino acids, the imino acid carrier and the taurine carrier. The imino acid carrier [7] is the principal carrier of proline and the sole carrier of sarcosine, β-alanine and γ-aminobutyric acid. Transport of these amino acids is characterized by $K_{1/2}$ values between 10 and 20 mM and maximum rates of transport of approx. 5 μmol cm^{-2} h^{-1}. In addition, like the small intestine of guinea-pig and rabbit, rat small intestine is equipped with a chloride- and sodium-dependent carrier of taurine with $K_{1/2}$ values in the range of 10–50 μM and maximum rates of transport between 20 and 50 nmol cm^{-2} h^{-1} [1,5,9,10].

The kinetics of baclofen absorption by rat small intestine have been estimated from the rate of baclofen disappearance from the intestinal lumen in vivo [3]. The middle part of rat small intestine appears to transport baclofen by a saturable process characterized by a $K_{1/2}$ of 7 mM and a maximum transport rate of 13 μmol h^{-1} [3]. With the same technique β-alanine was characterized by a $K_{1/2}^{\beta\text{-Ala}}$ of 11 mM and a maximum rate of transport of 26 μmol h^{-1} [4]. β-Alanine eliminated 2/3 of the baclofen transport with a K_i of 2.5 mM [12].

These results suggested that baclofen might be transported by the imino acid carrier. It is the purpose of the present study to examine this possibility, to examine whether baclofen is subject to secondary active transport and to determine the nature of the β-alanine resistant transport.

Materials and Methods

Materials

Male albino rats with body weights of 200 g, female rabbits, 2500–3000 g, and female albino guinea-pigs, 400 g, were used. The animals were raised and maintained with free access to water and food. Rats and guinea-pigs were anaesthetized by intraperitoneal administered phenobarbital. Rabbits were killed by intravenous phenobarbital. The abdomen was opened and the small intestine taken out. The mid 20 cm of the total rat small intestine were used for mounting in the influx chamber, or it was cut into 3–4 mm long segments, which were used for measuring steady-state uptake. The following segments were used for the

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topographic survey: The proximal jejunum (adjacent to the ligament of Treitz), mid jejunum (midintestinal), distal jejunum (middle of distal half) and distal ileum (0–15 cm from the ileocecal junction). The distal 25 cm of rabbit ileum and the mid 40 cm of guinea-pig small intestine were used.

All solutions were made from a phosphate buffer (pH 7.4) with composition (mM): Na, 140; K, 8; Ca, 2.6; Mg, 1; Cl, 140; phosphate, 8; SO_4, 1; D-glucose, 5. Sodium-free solutions were prepared by substituting *N*-methyl-D-glucamine for sodium and chloride free conditions by substituting sodium isethionate for NaCl. The experiments were carried out at 37°C under aeration with 100% O_2. ^3H or ^{14}C-labeled amino acids and ^3H- or ^{14}C-labeled poly(ethylene glycol) (M_r 4000) were purchased from New England Nuclear.

Unidirectional influx across the brush-border membrane

The segments of intestine used were excised, opened along the mesenteric attachment, and rinsed in ice cold buffer. Each segment was mounted on a Lucite plate with the mucosal surface facing upwards, and a Lucite block was clamped on the top of the plate. In this way four mucosal areas of 0.62 cm^2 were exposed in the bottom of wells, where the solution was oxygenated and stirred by high rates of O_2-flow [13]. The use of two blocks allowed eight measurements from each rat. With guinea-pigs and rabbits 4 blocks were used allowing 16 measurements from each animal.

The tissues were incubated for 20 min with an amino-acid-free solution containing 5 mM glucose which was then withdrawn. The well and mucosal surface were gently wiped with soft paper to remove adherent incubation fluid before injection of the test solution. The incubation period of 0.5 min was terminated by aspiration of the incubation fluid and flushing of the well with an ice-cold 300 mM mannitol solution. The exposed tissues were then punched out, briefly rinsed in ice-cold mannitol solution, blotted on hard filter paper, and extracted for 18 h in 0.1 M HNO_3. This extract was analyzed in a liquid scintillation spectrometer. The content of ^3H- or ^{14}C-PEG-4000 was used to correct for extracellular contamination, and thus corrected, the content of ^{14}C-, respectively ^3H-activity was used to calculate the rate of influx across the brush-border membrane.

The transport across the brush-border membrane (J_{mc}) was measured at different concentrations, $[A]_m$, and at different inhibitor concentrations, $[I]_m$, in the mucosa-bathing solutions. It was assumed that the unidirectional influx could be described as the sum of saturable processes and diffusion:

$$J_{mc}^{A} = \frac{J_{max}[A]_m}{K_{1/2} + [A]_m + \frac{K_{1/2}[I]_m}{K_i}} + P[A] \quad (1)$$

where P is the diffusive permeability of A in cm h^{-1}. J_{mc} is given as nmol cm^{-2} (serosal area) h^{-1}. The apparent affinity constant, $K_{1/2}$, and the inhibition constant, K_i, are in mM. The uninhibited influx of baclofen was fitted to a process of diffusion by nonlinear least-square fitting (Sigmaplot 4.0) and the estimate evaluated by the Chi-square test with df = number of experimental points minus number of parameters estimated.

The K_i values were calculated from ratios between inhibited and uninhibited fluxes assuming that these are described by Eqn. 1 and that $K_i = K_{1/2}$. Since the diffusive contribution to J_{mc}^{Bacl} is unaffected by any competitive inhibitor, its relative magnitude will increase with increasing inhibitor concentration and estimates of the apparent K_i will increase unless the diffusive contribution is subtracted. But if J_{mc}^{Bacl} is overcorrected for diffusion K_i will decrease with increasing inhibitor concentration.

All influx results are pooled data from three to six rats. Errors on fluxes are S.E. with the number of observations in parentheses. The data were evaluated by the paired Student's t-test, the unpaired t-test being used for experiments with an uneven number of observations (self-inhibition study).

Steady-state mucosal uptake

Steady-state tissue uptake was measured in segments of the everted intestine using the method described for rat ileum [2] with ^3H-or ^{14}C-labelled PEG-4000 as a marker of the extracellular space. The everted segments (approx. 150 mg wet weight) were incubated with the appropriate amino acid in the oxygenated solution for 40–80 min at 37°C. After 40 min incubation estimates of extracellular space and intracellular accumulation remain constant for at least the next 40 min indicating that by 40 min [^3H]PEG-4000 has equilibrated with the whole extracellular space [11]. After the incubation the segments were distributed into three equally large portions, and the wet weight determined. Two portions were extracted for 18 h in 0.1 M HNO_3 and one was dried for 24 h at 105°C to estimate the dry weight fraction. The ^3H and ^{14}C activities of the extracts were determined and the intracellular concentration of amino acids calculated. The results represent pooled data from two rats.

Results

Influx of baclofen across the brush-border membrane of rat small intestine

Topography

Influx of baclofen (0.01 mM) was determined in paired experiments at four locations along the whole small intestine.

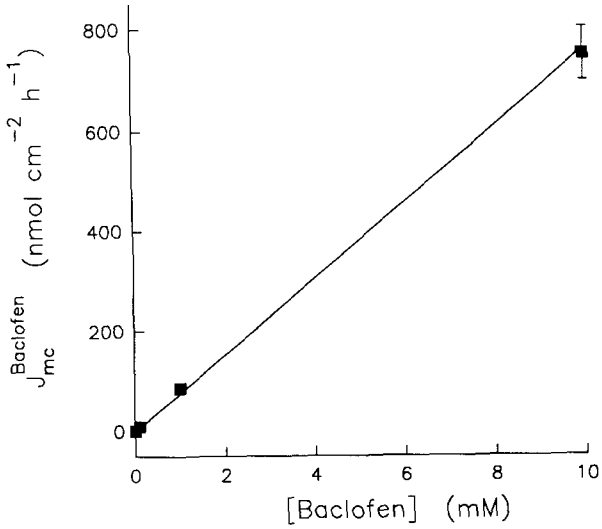

Fig. 1. Baclofen influx across the brush-border membrane of rat jejunum measured at 0.01, 1.0, 10.0 mM baclofen ($n = 13$) and at 0.1 mM baclofen ($n = 7$). Each point represents the mean ± S.E. The curve represents the best fit to transport by simple diffusion with a permeability coefficient of 0.076 ± 0.02 cm h^{-1} (chi-square = 9.921, $df = 3$, $P = 0.02$).

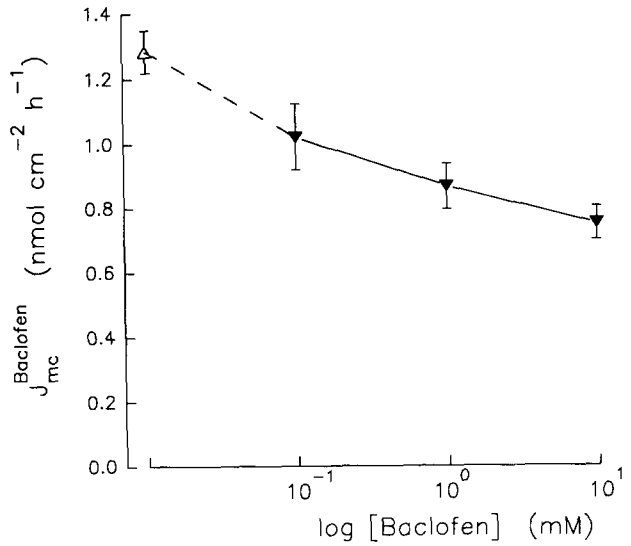

Fig. 2. Baclofen influx across the brush-border membrane of rat jejunum. Paired measurements at 0.01 mM baclofen alone (△, $n = 13$) and inhibited by 0.1 ($n = 7$; $p = 0.04$), 1.0 ($n = 13$; $p = 0.002$) or 10.0 mM ($n = 13$; $p = 10^{-6}$) baclofen (▼). The inhibition by 10 mM baclofen is larger than by 0.1 mM ($p = 0.017$) and not different from inhibition of 1.0 mM baclofen ($p = 0.21$). The inhibition at 1.0 mM is not different from inhibition at 0.1 mM ($p = 0.24$). Each point represents the mean ± S.E.

J_{mc}^{Bacl} was ($n = 6$) 0.882 ± 0.080 nmol cm^{-2} h^{-1}, 1.035 ± 0.124 and 1.133 ± 0.117 in proximal, mid and distal jejunum, respectively, and 1.214 ± 0.201 in distal ileum. This variation of J_{mc}^{Bacl} along the rat small intestine is statistically insignificant. The mid jejunum was chosen for this study because it has been employed in most previous studies of amino acid absorption in rat small intestine.

Self-inhibition

Paired measurements of J_{mc}^{Bacl} were made at 0.01, 0.1, 1.0 and 10.0 mM baclofen. As shown in Fig. 1, the results suggested a linear relationship between concentration and transport. The best fit by nonlinear regression analysis was to a process of diffusion with a permeability of 0.076 ± 0.002 cm h^{-1}; chi-square = 9.92, $df = 3$, $p = 0.02$. The residuals were caused by poor fits at 0.01 and 0.1 mM baclofen. Therefore, the observed fluxes from Fig. 1 were recalculated in order to examine for baclofen self-inhibition (Fig. 2). J_{mc}^{Bacl} measured at 0.01 mM baclofen was significantly inhibited by 0.1 ($p = 0.040$), 1.0 ($p = 0.002$) and 10 mM ($p = 10^{-6}$) baclofen. The degree of inhibition at 10 mM baclofen exceeds significantly the inhibition at 0.1 mM ($p = 0.017$) but not that at 1.0 mM ($p = 0.21$) baclofen. Thus, the data of Fig. 2 define 0.076 cm h^{-1} as the upper limit of the diffusive permeability of baclofen.

Following the arguments in the section on methods estimates of $K_{1/2}^{Bacl}$ for 0.1, 1.0 and 10 mM baclofen against J_{mc}^{Bacl} were made assuming a diffusive permeability of 0, 0.060, 0.073 and 0.074 cm h^{-1}. As shown in Table I these estimates indicate that $P = 0.073$ cm h^{-1} is the best choice. It corresponds to a $K_{1/2}^{Bacl}$ of 0.3 mM. With $K_{1/2} = K_i = 0.3$ mM and $P = 0.073$ cm h^{-1} the uninhibited baclofen flux of 1.28 nmol cm^{-2} h^{-1} at 0.01 mM baclofen this corresponds to a J_{max}^{Bacl} of 15 nmol cm^{-2} h^{-1}.

Mutual inhibition between baclofen and other amino acids

The apparently very low transport capacity and the relatively high affinity pointed to the Na^+- and Cl^--dependent taurine carrier [1,5], while the inhibitory effect of β-alanine suggested the imino acid carrier as responsible for baclofen transport [8]. In paired experiments, transport of baclofen was measured at 0.01 mM baclofen using 40 mM β-alanine, MeAIB, leucine, γ-aminobutyric acid, taurine, or lysine, or 10 mM baclofen as inhibitors. The results of these experiments are summarized in Table II. It is seen that β-alanine,

TABLE I

Baclofen self-inhibition of J_{mc}^{Bacl} measured at 0.01 mM baclofen in rat jejunum

P (cm h^{-1})	(I) (mM baclofen):	K_i^{Bacl} (mM)		
		0.1	1.0	10
0		0.38	2.11	14.1
0.06		0.15	0.65	2.8
0.073		0.10	0.32	0.35
0.074		0.10	0.31	0.18

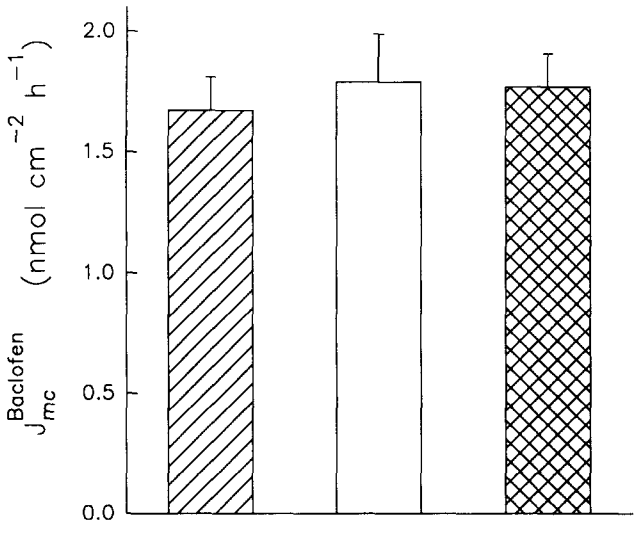

Fig. 3. Baclofen influx across the brush-border membrane of rat jejunum. Paired measurements at 0.01 mM baclofen in the presence of 140 mM NaCl (hatched, $n = 8$); 0 mM Na^+ (open, $n = 8$); and 0 mM Cl^- (cross hatched, $n = 6$). Errors are S.E. The differences are statistically insignificant.

leucine and γ-aminobutyric acid significantly inhibit the transport of baclofen, whereas taurine, MeAIB and lysine do not.

With this knowledge, baclofen was examined as a possible inhibitor of the transport of β-alanine and taurine both at 0.005 mM using 10 mM baclofen as inhibitor. The results (Table II) demonstrate that transport of both β-alanine and taurine is unaffected by the presence of baclofen.

These patterns of inhibition exclude the imino acid carrier, the taurine carrier and the carriers of cationic and neutral amino acids from further consideration as transporters of baclofen [6–8].

In paired experiments transport of baclofen at 0.005 mM was demonstrated to be both Na^+- and Cl^--independent (Fig. 3). The Na^+-dependence of β-alanine (0.005 mM) transport served as a control of the sensitivity of the experimental procedure: $J_{mc}^{\beta\text{-Ala}}$ was 1.284 ± 0.109 nmol cm^{-2} h^{-1} in the presence of Na^+ and 0.742 ± 0.081 ($n = 8$; $p < 0.01$) in the absence of Na^+.

Steady-state mucosal uptake of baclofen

The Na^+- and Cl^--independence of baclofen transport questioned the idea [12] that baclofen might be subject to secondary active transport in the rat small intestine. This question was addressed by paired measurements of steady-state uptake by rings of everted jejunum of baclofen at 0.01 mM using uptake of proline at 1 mM as control of tissue viability. For proline the steady-state ratio of distribution between the intracellular and extracellular spaces was 11 ± 0.6 ($n = 4$) while for baclofen it was 1.0 ± 0.1 ($n = 12$).

This evidence of passive, equilibrating transport could reflect extensive metabolism of baclofen with ^3H_2O as the final product. Therefore, a sac of everted jejunum (0.5 g wet weight) was incubated for 70 min in 8 ml with only tracer amount of baclofen (500 000 dpm 50 μl^{-1}). During the 70 min of incubation the volume of serosal fluid increased from 1.0 to 1.1 ml. After the incubation the activity of the mucosal fluid was 441 700 dpm 50 μl^{-1} and that of the serosal fluid 378 150 dpm 50 μl^{-1}. Identical activities were found when 50 μl samples were evaporated to dryness and redissolved in scintillation fluid for counting.

Finally, samples of mucosal and serosal fluids were examined by thin layer chromatography on Silica Gel GF using 2% acetic acid or n-butanol/acetic acid/water, (25 : 4 : 10), as solvent systems. In 2% acetic acid only 1 peak was detected. In the n-butanol/acetic acid/water system, secondary peaks with identical R_F values were observed both for the NEN-lot, the mucosal solution and the serosal solution, where the secondary peak amounted to respectively 2, 0.4 and 9% of the main peak. In the n-butanol/acetic acid/water system the R_F value for ^{14}C-γ-aminobutyric acid was

TABLE II

Baclofen transport and interaction with other amino acids in rat jejunum

Amino acid transported	Inhibitors:	Baclofen (10 mM)	Leucine (40 mM)	GABA (40 mM)	β-ala (40 mM)	MeAIB (40 mM)	Lysine (40 mM)	Taurine (40 mM)
Baclofen (0.01 mM)	+	0.65 ± 0.09 [a]	1.04 ± 0.07 [a]	1.11 ± 0.06 [b]	0.94 ± 0.10 [a]	1.68 ± 0.16 [c]	1.49 ± 0.04 [c]	1.79 ± 0.33 [c]
	−	1.41 ± 0.06	1.57 ± 0.07	1.67 ± 0.14	1.41 ± 0.06	1.88 ± 0.39	1.47 ± 0.08	1.88 ± 0.39
		(6)	(15)	(8)	(6)	(6)	(8)	(6)
β-alanine (0.005 mM)	+	2.16 ± 0.13 [c]						
	−	2.18 ± 0.12						
		(8)						
Taurine (0.005 mM)	+	0.61 ± 0.06 [c]						
	−	0.64 ± 0.02						
		(5)						

([a] $p < 0.001$; [b] $p < 0.01$; [c] not significant).

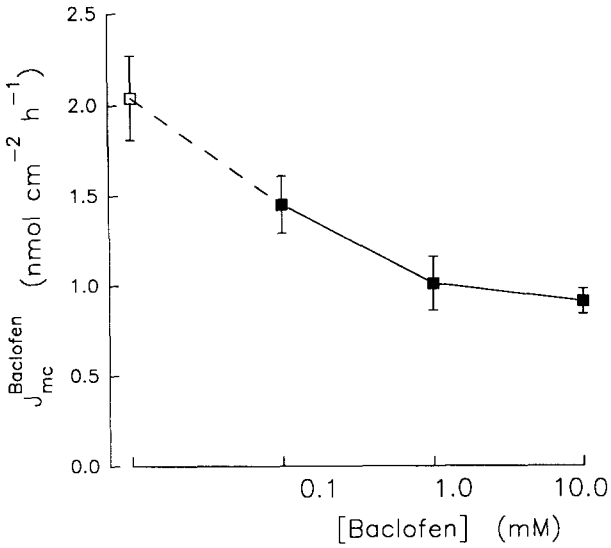

Fig. 4. Baclofen influx across the brush-border membrane of rabbit distal ileum. Paired measurements at 0.01 mM baclofen (□) inhibited by 0.1 (NS), 1.0 ($p = 0.067$) and 10.0 mM ($p = 0.009$) baclofen (■). Each point represents the mean ± S.E. of four observations. Assuming a passive permeability of 0.08 cm h^{-1} the data at 0.1, 1.0 and 10.0 mM baclofen correspond to $K_{1/2}^{Bacl}$ of 0.1, 0.2 and 0.1 mM, and a J_{max}^{Bacl} of 100 nmol cm^{-2} h^{-1}.

different from both ^{3}H-peaks. Thus, the lack of evidence for (secondary) active transport is not caused by metabolic transformation of the labelled baclofen.

Baclofen transport across guinea-pig and rabbit small intestine

The low rate of transport and its independence of Na^+ and Cl^- were surprising. With the purpose of examining whether these qualities were peculiar to the rat, baclofen transport was briefly studied in guinea-pig and rabbit small intestine. In the distal rabbit ileum J_{mc}^{Bacl} was measured at 0.01, 0.1, 1.0 and 10.0 mM baclofen. As for the rat, a relatively high passive permeability, a low maximum rate of transport and a $K_{1/2}$ well below 1 mM was indicated (Fig. 4). Paired experiments were performed in the guinea-pig jejunum examining the Na^+- and Cl^--dependence of J_{mc}^{Bacl} at 0.01 mM baclofen ($n = 4$) and $J_{mc}^{\beta\text{-Ala}}$ at 0.005 mM β-alanine ($n = 7$). J_{mc}^{Bacl} was 1.52 ± 0.20 nmol cm^{-2} h^{-1} against 1.32 ± 0.17 nmol cm^{-2} h^{-1} with and without sodium and 1.53 ± 0.05 against 1.34 ± 0.09 with and without chloride. $J_{mc}^{\beta\text{-Ala}}$ was 0.56 ± 0.05 nmol cm^{-2} h^{-1} against 0.26 ± 0.03 ($p < 0.01$) with and without sodium.

Discussion

Previous reports have described transport of baclofen by rat small intestine in vivo as a saturable process inhibitable by β-alanine [3,12]. It was the purpose of the present study to examine the transport of baclofen in more detail, and in particular to determine the pathways for its transport.

The results of measurements over a 1000-fold concentration range clearly demonstrated that, even at the low concentration of 0.01 mM, approx. 50% of the total transport of baclofen is by a nonsaturable pathway, probably diffusion. This, together with relatively large inter- and intraindividual variations, made it impossible to reach good estimates of the kinetic parameters of baclofen transport. However, the data of Figs. 1 and 2 suggest a value of $K_{1/2}^{Bacl}$ well below 1 mM and a passive permeability of approx. 0.07 cm h^{-1}. The smaller difference between the estimates of $K_{1/2}^{Bacl}$ for the 1 and 10 mM data obtained assuming a passive permeability of 0.073 rather than 0.060 and 0.074 cm h^{-1} (Table I) indicates that 0.073 is the better estimate and points to a maximum rate of transport of 10–20 nmol cm^{-2} h^{-1}.

The very large diffusive contribution to the transport of baclofen observed in vitro may in vivo be represented by the large β-alanine resistant fraction. The very low maximum rate of transport pointed to the Na^+- and Cl^--dependent taurine carrier as the means of baclofen transport [1]. However, lack of mutual inhibition between baclofen and taurine made this an untenable proposal. The inhibitory effect of β-alanine and γ-aminobutyric acid pointed to the imino acid carrier as responsible for the saturable fraction of J_{mc}^{Bacl}. However, this possibility had to be rejected, since MeAIB was not an inhibitor of baclofen transport. Likewise, the lack of inhibition by lysine excludes the carrier of cationic amino acids, while the inhibitory effect of β-alanine excludes the carrier of α-amino-monocarboxylic acids [12].

The independence of sodium and chloride indicated that baclofen is not subject to secondary active transport. This was confirmed by a ratio of one for the steady-state distribution ratio of 0.01 mM baclofen between tissue intracellular space and incubation fluid.

In conclusion, intestinal transport of baclofen is the sum of a large diffusive contribution and contribution by a passive saturable process with a very low capacity and a high affinity.

Acknowledgements

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