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Characterisation of penicillin-G uptake in rabbit small-intestinal brush-border membrane vesicles

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Abstract

Uptake of penicillin-G has been studied in rabbit intestinal brush-border membrane vesicles (BBMV). Penicillin-G was transported into the lumen of BBMV via an H^+-dependent, Na^+-independent uptake system. This was a saturable carrier-mediated process, which adhered to Michaelis-Menten kinetics, having a pH optimum of 4.5 and resulting in a net-negative charge transfer. V_{max} was 59 nmol penicillin-G (mg protein)$^{-1}$ (30 s)$^{-1}$ and K_m 22.7 mM. Ampicillin, penicillin-V, cefadroxil, cephalexin, cephalothin, cephradine, L-carnosine, glycyl-L-alanine, glycyl-L-tyrosine and glycylglycylglycine inhibited the uptake of penicillin-G. However, glycylsarcosine stimulated uptake by 92%. Countertransport experiments suggested that this effect took place at the active site of the transporter. Penicillin-G uptake appeared to be mediated via a common transport system shared by penicillins, cephalosporins and peptides.

Keywords: Brush-border membrane vesicle; β-Lactam; Antibiotic uptake; Penicillin-G; Oral administration; pH gradient

1. Introduction

Not all β-lactam antibiotics are orally absorbed, but the precise reasons for this are as yet not known. They are hydrophilic weak acids containing a peptide bond and are completely ionised at the pH of the intestinal contents. On the basis of pH-partition theory and their chemical and physicochemical properties [1], β-lactam antibiotics would be expected to be poorly absorbed from the small intestine. However, pharmacokinetic studies showed [2] that many of them were well absorbed.

Passive diffusion through the paracellular pathway appeared to account only for a small amount of orally absorbed β-lactams [3]. It appeared that they were actively transported across the brush-border membrane (BBM) of the enterocyte via a proton-dependent peptide carrier [4–13] and subsequently exited into the bloodstream via the basolateral membrane. Early studies showed that the transport of some hydrolysis-resistant di- and tripeptides (e.g., glycylsarcosylsarcosine, L-carnosine, glycylsarcosine) was an active energy-requiring process [14,15]. Subsequent studies in brush-border membrane vesicles (BBMV) showed that this process was carrier-mediated, saturable, adhered to Michaelis-Menten kinetics and could be competitively inhibited by other di- and tripeptides and aminocephalosporins [6,16–20]. Furthermore, peptide transport was shown to be stereospecific, independent from amino acid transport, and that hydrolysis and transport were distinct processes [4,10,21].

In 1985, Ganapathy and Leibach [21] postulated that peptides were co-transported with H^+. The transport of such ions caused a depolarisation of the membrane potential and led to a net transfer of positive charge [18,19]. The H^+-gradient across the membrane of the intact intestinal cell is thought to be maintained by the Na^+/H^+ exchanger located in the BBM and the Na^+/K^+ ATPase in the basolateral membrane. Thus, peptide transport has been described as a 'tertiary active' transport system.

Although it was thought that the α-amino acid at position 7 of the cephalosporin skeleton represented a key feature for the active transport of aminocephalosporins, recent studies have shown that cephalosporins lacking this

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group, such as cefixime, FK089 and ceftibuten, were transported across the membrane of BBMVs [5–7,9–12,22]. The lack of the α-amino acid at position 7 makes these compounds anionic at physiological pH compared to other β-lactam antibiotics which are zwitterions at this pH [1,9]. However, the pH microclimate at the BBM has been shown to be acidic [23], influencing the ionisation and subsequently the transport of peptides and β-lactams.

Small-intestinal H^+/peptide carriers for human (hPepT1) [24] and rabbit small intestine (Pept1) [25] have recently been cloned. Another H^+/peptide carrier, HPT-1, cloned from the human colorectal cell line (Caco-2) appears to have a very different structure and might be associated with binding rather than transport functions, as it has been suggested to be related to the cadherin superfamily [26]. However, all of these carriers were able to mediate peptide and β-lactam uptake in a proton-gradient-dependent manner.

The latest member of the H^+/peptide cotransport family, PepT2, has been cloned from human kidney [27]. This transporter was distinct from that cloned from the human small intestine, displaying a 50% identity and a 70% homology and is not present in the human small intestine [27]. hPepT1 the human small-intestinal H^+/peptide cotransporter was only marginally detectable in human kidney tissue [24].

It has now been established that many β-lactam antibiotics are taken up in the small intestine by an H^+-driven peptide transport system [4–13,22,24,25] of which there may be more than one [6,7,10,13,16,22,24–26].

Penicillin [4] and one of its derivatives (ampicillin) [28] have as yet only been used as inhibitors or markers for the potential small-intestinal peptide transporter(s). To our knowledge there are no studies of the characteristics of small-intestinal uptake of penicillin itself. In the present paper we have characterised the uptake and inhibition of penicillin-G transport and suggest its potential importance for the design of orally active β-lactam antibiotics.

2. Materials and methods

2.1. Materials

Mes, Hepes, EDTA, FCCP, valinomycin, cefotaxime, cephalothin and all peptides and amino acids were obtained from Fluka (Dorset, UK). Ampicillin was from Aldrich (Dorset, UK), penicillin-G, penicillin V, amoxicillin, cephalexin, cefuroxime, cephradine, cephadroxil, probenecid, Tris and DIDS from Sigma (Dorset, UK). BCA-protein assay was from Pierce Chemicals (Chester, UK) and glucose test kit from Merck (Darmstadt, Germany). Radiochemicals, benzyl[^{14}C]penicillin potassium (specific activity 59 mCi/mmol) and D-[U-^{14}C]glucose (spec. act. 292 mCi/mmol) were from Amersham (UK). Scintillation liquid 'OptiPhase safe' was from Wallac (Milton Keynes, UK). All other chemicals were of the highest purity available.

2.2. Animals

New Zealand White male rabbits (2–2.5 kg) (Regal Rabbits, London, UK) were kept on a standard diet and water ad libitum until death.

2.3. Preparation of brush-border membrane vesicles from rabbit small intestine

Rabbits were killed by cervical dislocation and approx. 1 m of the proximal small intestine was removed, washed in ice-cold 0.9% NaCl, everted on a glass rod, cut into 2 cm strips, snap-frozen in liquid nitrogen and stored at −80°C until use. Brush-border membrane vesicles from rabbit small intestine were prepared according to a modification of the salt precipitation method described by Kessler et al. [29] and Shirazi-Beechey et al. [30]. All the following steps were carried out at 4°C. On the day of use, the everted and frozen intestine was slowly defrosted, suspended in 10 mM Hepes/Tris, 40 mM mannitol at pH 7.1 and mixed with a 'Vibromixer Type E1' (Chemap, Volketswil, Switzerland) at maximal output for 10 min. After coarse filtration, the suspension was further homogenised using an Ultra-Turrax mixer (IKA-Werk, Germany) at maximal output for 5 min. $MgCl_2$ (final concentration 10 mM) was added to the homogenate which was stirred for 20 min and then centrifuged at 4000 × *g* for 15 min. The supernatant was then centrifuged at 27 000 × *g* for 30 min. The resulting pellet was resuspended in 10 mM Hepes/Tris, 0.1 mM $MgSO_4$, 100 mM mannitol at pH 7.1 using a 23 G needle and a 5 ml syringe, and the volume was adjusted to 200 ml. After centrifugation at 4000 × *g* for 15 min the supernatant was centrifuged at 27 000 × *g* for 30 min. The pellet containing the BBMV was then resuspended using a 23 G needle, as before, in 50 ml of loading buffer, as indicated for each experimental set, and centrifuged at 27 000 × *g* for 30 min. The final pellet was resuspended in 5 ml of loading buffer required for the individual experimental sets by passing through a 23 G hypodermic needle several times. No loss of enzyme or transport activity was detected upon storage of BBMV in liquid nitrogen up to 18 weeks after preparation.

2.4. Characterisation of brush-border membrane vesicles

The brush-border membrane marker enzymes K^+-activated phosphatase [31], alkaline phosphatase [32], aminopeptidase N [33] and sucrase [34] were assayed by standard methods. Glucose liberated by sucrase was assayed using a commercial kit (Glucose GOD-PAP method, Merckotest 14365, Merck Diagnostica, Germany). Protein concentration was determined using a commercial kit based on the bicinchonic acid (BCA) method [35]. The integrity

of the BBMV was routinely tested by their ability to accumulate radiolabelled glucose in response to an Na^+-gradient, as described previously [36].

2.5. Glucose uptake measurements

D-[U-^{14}C]Glucose uptake was carried out as previously described [33] with slight alterations. The loading buffer of the vesicles was 5 mM Hepes/Tris, 100 mM mannitol, 100 mM KCl at pH 7.1. The assay buffer consisted of 100 mM mannitol, 100 mM NaCl (or 100 mM NaSCN), 5 mM Hepes/Tris at pH 7.1 and a final D-[U-^{14}C]glucose concentration of 0.1 mM. Passive uptake was measured using assay buffer containing 100 mM KCl instead of 100 mM NaCl. The transport studies were carried out at 37°C by mixing 10 μl of vesicles with 100 μl of assay mix. The reaction was stopped by adding 1 ml of 150 mM KCl, 5 mM Hepes/Tris (pH 7.1) at 4°C and 1 ml of each assay was rapidly filtered through GSWP 0.22 μm filters (Millipore, Watford, UK). After washing with 10 ml of ice-cold 150 mM KCl, 5 mM Hepes/Tris (pH 7.1), the filters were dissolved in 4 ml of scintillation liquid and counted in a LKB 1219 Rackbeta (LKB, UK). The samples were corrected for reagent blank (non-specific binding to filters) and vesicle blank (zero-time uptake) allowing for non-specific binding to vesicles.

2.6. Penicillin-G uptake

Penicillin-G uptake was carried out as above with the following modifications; vesicles were loaded with 5 mM Hepes/Tris, 0.1 mM $MgSO_4$, 100 mM mannitol, 100 mM KCl at pH 7.1; ^{14}C-labelled penicillin-G was diluted 1:10 with unlabelled penicillin-G to give a final concentration of 0.1 mM in assay mix containing 100 mM KCl, 100 mM mannitol, 5 mM Mes/Tris for pH 5.0, or 5 mM Hepes/Tris for pH 7.1, respectively; samples were incubated at 37°C over a range of time points; stopping and washing buffer was 150 mM NaCl, 5 mM Hepes/Tris (pH 7.1) at 4°C. All the following experiments were carried out according to the same assay principle.

2.7. Concentration dependency of penicillin-G uptake

Penicillin-G concentration was increased by addition of unlabelled penicillin-G. Changes in osmolarity due to increased penicillin-G concentration were corrected for by decreasing the mannitol concentration accordingly. Assay conditions are described in Fig. 2. Stopping and washing buffer was 125 mM NaCl, 50 mM Hepes/Tris (pH 7.1) at 4°C.

2.8. pH optimum of penicillin-G uptake

The assay conditions are given in Fig. 3. Stopping and washing buffer was 125 mM NaCl, 50 mM Hepes/Tris (pH 7.1) at 4°C.

Table 1
Effect of various β-lactam, peptides and amino acids on penicillin-G uptake

Inhibitor (10 mM)	% Uptake	±S.D. (%)	*P*
Control (Pen-G 0.1 mM)	100	5	
Amoxicillin	86	6	< 0.001
Ampicillin	42	9	< 0.001
Penicillin-V	43	10	< 0.001
Penicillin-G	64	9	< 0.001
Cefadroxil	58	10	< 0.001
Cephalexin	33	14	< 0.001
Cephalothin	71	18	< 0.001
Cephradine	82	5	< 0.001
Cefotaxime	109	9	< 0.02
Cefuroxime	104	18	> 0.05
L-Carnosine	65	6	< 0.001
Gly-L-Ala	48	7	< 0.001
Gly-L-Tyr	52	13	< 0.001
Gly-Gly	82	12	< 0.001
Gly-Sar	192	8	< 0.001
Gly-Gly-Gly	44	2	< 0.001
Gly	88	15	< 0.05
Sar	61	11	< 0.001
Gly + Sar	75	6	< 0.001
Probenecid (3 mM)	63	7	< 0.001

The external buffer contained 50 mM Mes/Tris (pH 5.0), 100 mM KCl, 40 mM mannitol (30 mM for glycine + sarcosine, 50 mM for control), 10 mM inhibitor and 0.1 mM penicillin-G. The vesicles were loaded with 50 mM mannitol, 100 mM KCl, 50 mM Hepes/Tris (pH 7.1) and 0.1 mM $MgSO_4$. The samples were incubated for 30 s at 37°C. Stopping and washing buffer was 125 mM NaCl, 50 mM Hepes/Tris (pH 7.1) at 4°C. The results represent the average of three distinct experiments carried out in triplicate. *P* values were calculated using the Student *t*-test.

2.9. Effect of osmolarity on penicillin-G uptake

Osmolarity of the incubation medium was increased by addition of mannitol. Assay conditions are given in Fig. 4. Samples were incubated for 30 min at 37°C to ensure that the reaction was at equilibrium. Stopping and washing buffer was 125 mM NaCl, 50 mM Hepes/Tris (pH 7.1) at 4°C.

2.10. Inhibition of penicillin-G uptake

Assay conditions are described in Table 1. Stopping and washing buffer was 125 mM NaCl, 50 mM Hepes/Tris (pH 7.1) at 4°C. Competitive inhibition and counter transport experiments were carried out as outlined in Figs. 5 and 6, respectively.

2.11. Effect of membrane potential on penicillin-G uptake

Assays were carried out at 25°C for 30 s under the conditions as stated in Figs. 7–9. Stopping and washing buffer was 250 mM mannitol, 50 mM Hepes/Tris (pH 7.1) at 4°C.

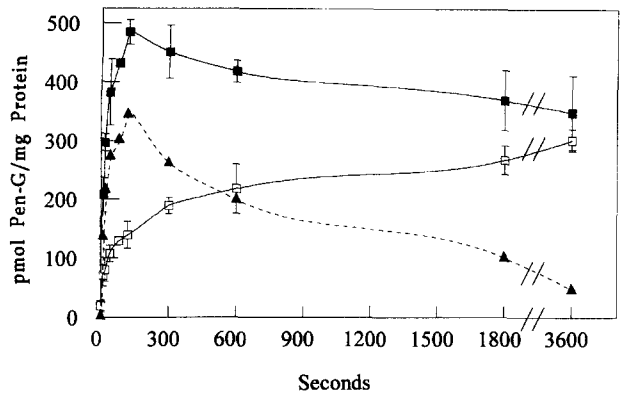

Fig. 1. Time-course of penicillin-G uptake in rabbit intestinal BBMV. Vesicles were loaded with 5 mM Hepes/Tris, 0.1 mM $MgSO_4$, 100 mM mannitol, 100 mM KCl at pH 7.1. Assay mix contained 100 mM KCl, 100 mM mannitol, 0.1 mM penicillin-G and 5 mM Mes/Tris for pH 5.0 (■——■), or 5 mM Hepes/Tris for pH 7.1 (□——□), respectively. The samples were incubated at 37°C. Active transport is represented as Δ pH = pH 5.0 − pH 7.1 (▲---▲). The results represent the average of three distinct experiments carried out in triplicate. Error bars represent standard deviation (±S.D).

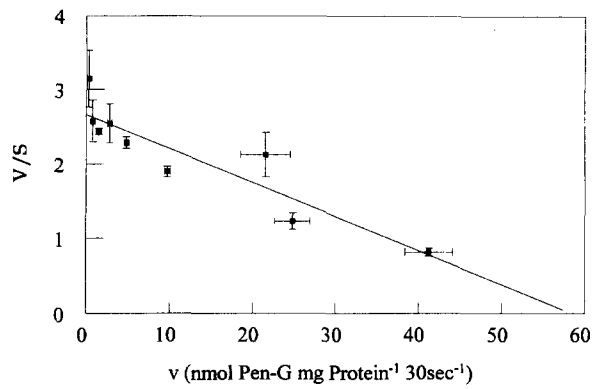

Fig. 2. Eadie-Hofstee plot of penicillin-G uptake in rabbit intestinal BBMV. Vesicles were loaded with 50 mM Hepes/Tris, 0.1 mM $MgSO_4$, 50 mM mannitol, 100 mM KCl at pH 7.1. The external buffer contained 100 mM KCl, 0–50 mM mannitol (to correct for penicillin-G addition), 50 mM Mes/Tris (pH 5.0) and 0.1–50 mM penicillin-G . Samples were incubated for 30 s at 37°C. The results represent the average of three distinct experiments carried out in triplicate. Error bars represent ±S.D. $r = 0.830$.

3. Results

3.1. Vesicle characterisation and glucose uptake

The purification factors of the marker enzymes were determined in three separate experiments in triplicate and compared well with results achieved by other groups [29,30]. The enrichment factors were for K^+-activated phosphatase 0.56 (S.D. ± 0.53), alkaline phosphatase. 8.96 (±0.95), aminopeptidase N 10.34 (±2.20) and sucrase 11.95 (±0.53). At 30 s under a 100 mM NaCl-gradient glucose transport showed an overshoot 20-fold compared to the uptake at equilibrium. Under a 100 mM NaSCN gradient the maximal observed overshoot was at 15 s with a 55-fold uptake compared to equilibrium values. These results compare well with these of other groups [30].

3.2. Penicillin-G transport

Under a pH gradient ($pH_{outside} < pH_{inside}$) penicillin-G exhibited the characteristic overshoot phenomenon (Fig. 1), associated with active transport, as shown by many other studies on β-lactams, peptides and peptide analogues [5–7,9,13,22,28]. Peak uptake of 485 pmol penicillin-G (mg protein)$^{-1}$ was achieved after 2 min and equilibrium was reached after 30 min. This compares well with the results of other groups using aminocephalosporins [5–7,9,13,22,28]. The uptake of penicillin-G in absence of a pH-gradient ($pH_{outside} = pH_{inside}$) increased steadily until reaching equilibrium after 60 min. The initial uptake of penicillin-G (Fig. 1) was largely due to active transport (Δ pH 5.0–pH 7.1) and the rate reduced drastically after 2 min. The active component of the uptake process conformed to Michaelis-Menten kinetics: V_{max} 59 nmol penicillin-G (mg protein)$^{-1}$ (30 s)$^{-1}$, K_m 22.7 mM (Fig. 2) and had a pH optimum of 4.5 (Fig. 3).

3.3. Separation of uptake and binding

Extrapolation to infinite osmolarity of the line relating equilibrium uptake to the reciprocal of the osmolarity of the external buffer suggested that more than 85% of penicillin-G was transported into the vesicular space (Fig. 4). FCCP (0.05 mM) reduced the active component of penicillin-G uptake by 28% (S.D. ± 4%) under a 50 mM KCl gradient and by 50% (±5%) under a 50 mM KSCN gradient, assuming that the KSCN result in absence of FCCP represented 100% uptake (see Fig. 8). A similar result was found by Tsuji et al. [5] using cefixime.

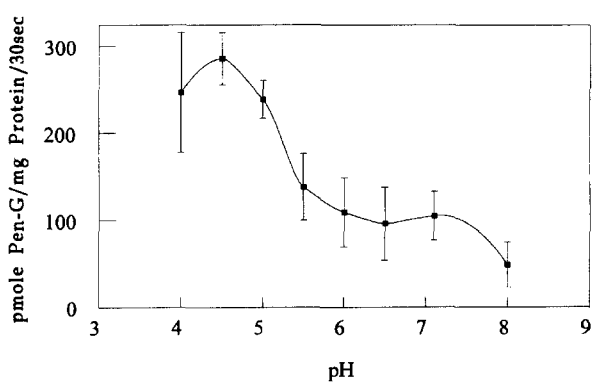

Fig. 3. Effect of pH on penicillin-G uptake in rabbit intestinal BBMV. Vesicles were loaded with 50 mM Hepes/Tris, 0.1 mM $MgSO_4$, 50 mM mannitol, 100 mM KCl at pH 7.1. The external buffer was 50 mM Mes/Tris (pH 4.0–6.5) or 50 mM Hepes/Tris (pH 7.1 and 8.0), 100 mM KCl, 50 mM mannitol and 0.1 mM penicillin-G. The samples were incubated for 30 s at 37°C. The results represent the average of three distinct experiments carried out in triplicate. Error bars represent ±S.D.

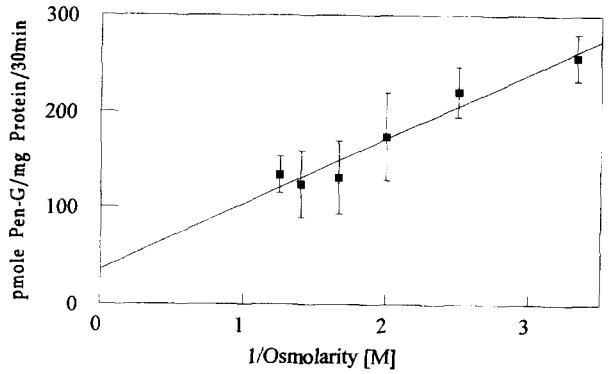

Fig. 4. Effect of osmolarity on penicillin-G uptake in rabbit intestinal BBMV. Vesicles were loaded with 50 mM Hepes/Tris, 0.1 mM $MgSO_4$, 50 mM mannitol, 100 mM KCl at pH 7.1. The external buffer contained 100 mM KCl, 50–550 mM mannitol, 50 mM Mes/Tris (pH 5.0) and 0.1 mM penicillin-G. Samples were incubated for 30 minutes at 37°C. The results represent the average of three distinct experiments carried out in triplicate. Error bars represent ±S.D. $r = 0.945$.

3.4. Inhibition of penicillin-G transport

Penicillin-G uptake was inhibited by: penicillin V, ampicillin, cephalothin, cephalexin, cephradine and cephadroxil (see Table 1). The two poorly absorbed cephalosporins, cefuroxime and cefotaxime, had no inhibitory effect. Kinetic plots indicated that cephalexin was a competitive inhibitor of penicillin-G uptake (Fig. 5).

Of the peptides used, L-carnosine, glycyl-L-tyrosine, glycylglycine, glycyl-L-alanine and glycylglycylglycine inhibited penicillin-G uptake (Table 1). However, glycylsarcosine stimulated penicillin-G uptake by 92%. Sugawara et al. [13] have reported a 30% stimulation of cephalexin uptake by glycylsarcosine. The action is thought to take

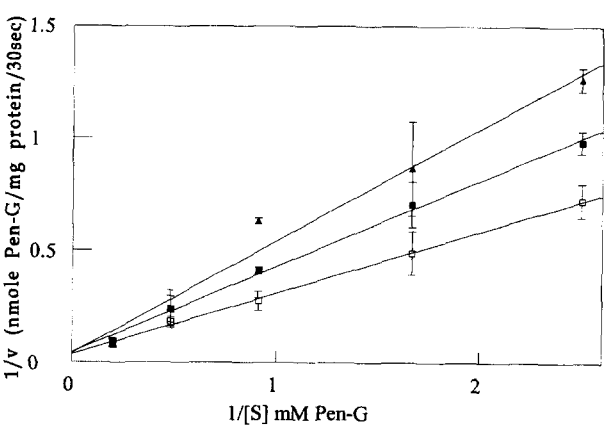

Fig. 5. Effect of 0 (□ ——— □, $r = 0.998$), 5 (■ ——— ■, $r = 0.996$) and 10 mM (▲ ——— ▲, $r = 0.975$) cephalexin on penicillin-G transport in rabbit intestinal BBMV. The external buffer contained 50 mM Mes/Tris (pH 5.0), 100 mM KCl, 35–49.7 mM mannitol, 0 mM, 5 mM, 10 mM cephalexin, respectively, and 0.4 to 5.1 mM penicillin-G. Vesicles were loaded with 50 mM Hepes/Tris, 0.1 mM $MgSO_4$, 50 mM mannitol, 100 mM KCl at pH 7.1. The samples were incubated for 30 s at 37°C. The results represent the average of three distinct experiments carried out in triplicate. Error bars represent ±S.D.

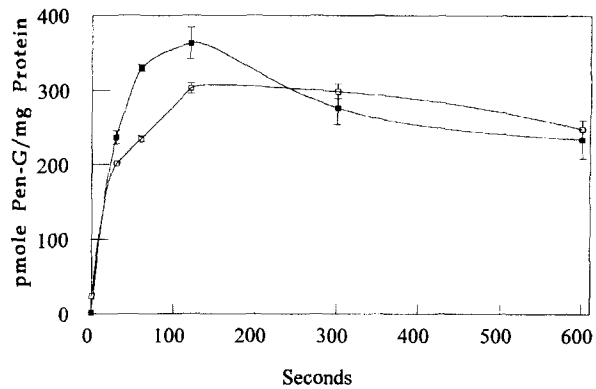

Fig. 6. Counter transport effect of 10 mM glycylsarcosine on penicillin-G uptake in rabbit intestinal BBMV. The external buffer for the controls contained 50 mM Mes/Tris (pH 5.0), 100 mM KCl, 50 mM mannitol, 1 mM glycylsarcosine and 0.1 mM penicillin-G (■ ——— ■). Vesicles for the controls were loaded with 50 mM Hepes/Tris, 0.1 mM $MgSO_4$, 50 mM mannitol, 100 mM KCl at pH 7.1. The external buffer for the counter transport contained 50 mM Mes/Tris (pH 5.0), 100 mM KCl, 50 mM and 0.1 mM penicillin-G (□ ——— □). Vesicles for the counter transport were loaded with 50 mM Hepes/Tris, 0.1 mM $MgSO_4$, 50 mM mannitol, 100 mM KCl at pH 7.1 and 10 mM glycylsarcosine. The samples were incubated for 30 s at 37°C. The results represent the average of three distinct experiments carried out in triplicate. Error bars represent ±S.D.

place at the active site of the transporter, since efflux of glycylsarcosine from preloaded BBMV reduced the uptake of penicillin-G (Fig. 6).

The two amino acids, glycine and sarcosine, gave different results (Table 1). Glycine did not inhibit penicillin-G uptake very well, which agreed with reports that peptide and β-lactam antibiotic transport was independent of amino acid transport [5,7,10,11,37]. However, sarcosine gave an inhibition of 40%. When using equimolar quantities (10 mM) of glycine and sarcosine together, an inhibition of 25% was observed. The reasons for this latter result were unclear.

The addition of probenecid (3 mM), an inhibitor of penicillin excretion in the kidney, inhibited penicillin-G uptake by approximately 40% (Table 1). This suggests that the transporters responsible for uptake in the small intestine and secretion in the renal tubule share some common features, although transport is mediated by two distinct transporters [24,27].

3.5. Effect of membrane potential on penicillin-G uptake

Vesicles were loaded with mannitol and Hepes/Tris. The electrochemical gradient was altered by varying the composition of the (external) ion gradients across the vesicular membrane. Changing the cation gradient (50 mM) from K^+ to Na^+ led to a reduction of 20% (S.D. ± 4%) of penicillin-G uptake, irrespective of the anion present, showing that penicillin uptake was not energised by an Na^+ gradient. Compared to the uptake of penicillin-G under the influence of a proton gradient alone, expressed

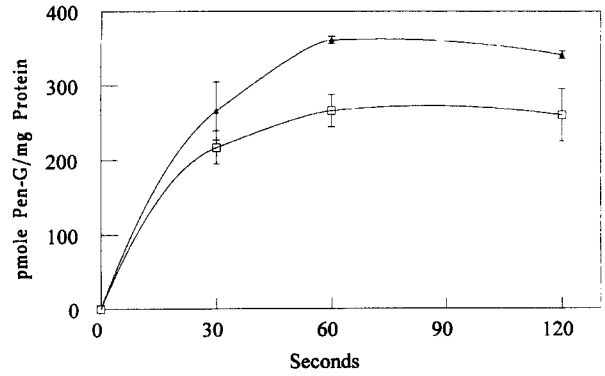

Fig. 7. Effect of 1 μM valinomycin on penicillin-G uptake in rabbit intestinal BBMV. The external buffer contained 50 mM Mes/Tris (pH 5.0), 250 mM mannitol, 0.1 mM penicillin-G. The vesicles were loaded with 50 mM mannitol, 100 mM KCl, 50 mM Hepes/Tris (pH 7.1) and 0.1 mM $MgSO_4$. Valinomycin (□ ——— □) in ethanol was added to the vesicles to give a final concentration of 1 μM (in assay) and incubated for 5 min at 25°C prior to use. Control (▲ ——— ▲) samples were incubated as valinomycin samples in the presence of 0.5% ethanol. The samples were incubated at 25°C. The results represent the average of three distinct experiments carried out in triplicate. Error bars represent ±S.D.

as 100%, changes in the anion composition led to dramatic changes in penicillin-G uptake (see Fig. 9). The more permeable the anion, the greater the reduction in uptake: SO_4^{2-}, −14% (S.D.± 11%), Cl^-, −20% (±5%) and SCN^-, −52% (±4%), independent of the cation (Na^+ or K^+) present. Penicillin-G uptake thus appeared to result in a net negative charge transfer. Similar results were observed in studies using cefixime [5].

Furthermore, collapse of a K^+ gradient (K^+_{inside} > $K^+_{outside}$) using valinomycin greatly reduced uptake compared to control (Fig. 7).

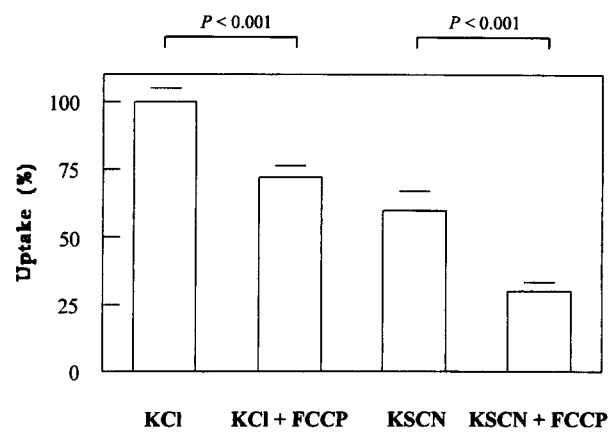

Fig. 8. Effect of 0.05 mM FCCP on penicillin-G uptake in rabbit small intestinal BBMV. The internal buffer was 50 mM Hepes/Tris, 250 mM mannitol, at pH 7.1. BBMV were preincubated with either 0.5 mM FCCP (final concentration of 0.05 mM) and 0.5% ethanol or 0.5% ethanol (control) at 25°C for 5 min prior to use. The external buffer was 50 mM Mes/Tris (pH 5.0), 150 mM mannitol, 50 mM KCl (or KSCN), 0.1 mM penicillin-G. Samples were incubated for 30 s at 25°C. The results represent the average of three distinct experiments carried out in triplicate. Error bars represent ±S.D. P values were calculated using the Student t-test.

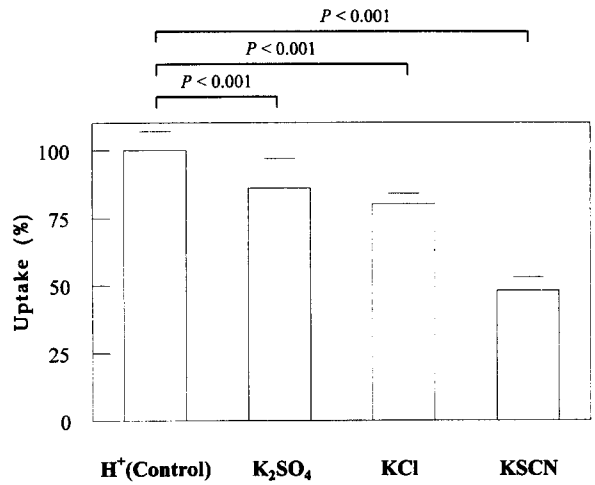

Fig. 9. Effect of anion displacement on penicillin-G uptake in rabbit small intestinal BBMV. The external buffer contained 50 mM Mes/Tris (pH 5.0), 150 mM mannitol (100 mM for K_2SO_4), 0.1 mM penicillin-G, 0.5% ethanol and 50 mM K_2SO_4, KCl or KSCN, respectively. The vesicles were loaded with 250 mM mannitol, 50 mM Hepes/Tris (pH 7.1) and 0.1 mM $MgSO_4$. The samples were incubated for 30 s at 25°C. The results represent the average of three distinct experiments carried out in triplicate. Error bars represent ±S.D. P values were calculated using the Student t-test.

4. Discussion

Penicillin-G has been shown not to be bioavailable in man due to instability in the strongly acidic environment of the stomach. Penicillin-V is acid-stable and can be dosed orally. Penicillin-G was used in the present studies since penicillin-V and other penicillins were not available in an isotopically labelled form.

The structural differences between penicillin-G and penicillin-V are trivial and do not involve the position traditionally associated with intestinal transport of β-lactam antibiotics. The fact that penicillin-V inhibited penicillin-G uptake suggests that they share the same carrier.

When β-lactam antibiotics and peptides were used as inhibitors of penicillin-G uptake it was found that the standard buffer concentration of 10 mM Mes/Tris for pH 5.0, as used by other groups [5–7,9–13,22,28], was too low to allow for the buffer capacity of the individual inhibitors. All the inhibitors used were by nature buffers themselves, which could have an effect on the imposed proton gradient. Thus, we used buffer concentrations of 50 mM.

The purified vesicles were enriched in BBM marker enzymes and no purification of basolateral marker enzymes was observed. The glucose transport studies showed that the BBMV used in these experiments have intact transport capabilities. The initial uptake rate of penicillin-G was markedly stimulated by the presence of an H^+ gradi-

ent. Collapsing the H^+ gradient with FCCP led to a reduction of penicillin-G uptake by almost 30% or 50%, depending on the anion present, indicating that penicillin-G uptake is driven by a proton-gradient-dependent mechanism. The large majority of initial penicillin-G uptake was via an active transport system, which was saturable, and displayed Michaelis-Menten kinetics. The response to osmolar shrinkage suggested that more than 85% of penicillin-G was taken up into the lumen of the vesicles rather than being bound to the membrane.

Ion substitution experiments suggested that the membrane potential is of importance. It has been previously reported that peptide [18,19] and cephalosporin [21] uptake was linked to a net positive charge transfer. Our studies suggest that penicillin-G uptake resulted in a net negative charge transfer, similar to that previously observed with cefixime [5]. This difference is probably because cefixime and penicillin-G are negatively charged (anionic) at pH 5.0 compared to the other peptides and cephaloporins tested [8,18,19] which are neutral (zwitterions). This suggests that the net negative charge transfer was a result of the uptake of the anionic form of penicillin-G. These results were further substantiated by using valinomycin. In vesicles loaded with KCl, collapse of the resulting K^+ gradient ($K^+_{inside} > K^+_{outside}$) reduced penicillin-G uptake due to the resulting interior negative charge.

Of the orally active β-lactam antibiotics tested for their inhibitory effects, ampicillin, penicillin-V, cefadroxil, cephalexin and cephalothin gave inhibitory effects ranging from 30% to almost 70%. Although amoxicillin was twice as effectively absorbed in man than ampicillin [38], hardly any inhibitory effect was observed. A similar result was found for cephradine. There is some evidence [7] that cephradine has different transport characteristics or even a different uptake system from cefixime. The presence of different transporters might explain the only slight inhibitory effect of either amoxicillin or cephradine on penicillin-G uptake. However, this warrants further investigation. Of the peptides used, L-carnosine, glycyl-L-alanine, glycyl-L-tyrosine and glycylglycylglycine showed inhibitory effects ranging from 35% to 65%. Glycylglycine displayed hardly any inhibitory effects, whereas glycylsarcosine stimulated uptake by over 90%. A less marked stimulatory effect (35%) of glycylsarcosine has been reported in experiments using cephalexin [13]. Cephalexin was by far the most potent inhibitor (67%) of penicillin-G uptake; either structural analogy or net charge could be of importance for this phenomenon. Since sarcosine is a derivative of glycine, the only slight inhibitory effect of glycylglycine compared to the other peptides could be explained in terms of structural similarity between glycylsarcosine and glycylglycine. Counter transport studies showed that glycylsarcosine efflux from BBMV inhibited the uptake of penicillin, indicating that the interaction was likely to take place at the active site of the transporter. However, the characteristics and the mechanism of this stimulation are as yet not understood and are currently under further investigation.

In conclusion, penicillin-G uptake in rabbit small-intestinal BBMV was mediated by a proton-gradient-driven, saturable active transport system resulting in a net negative charge transfer which can be inhibited by various β-lactams and dipeptides, and stimulated by glycylsarcosine. These findings may be of importance in the administration of orally active β-lactam antibiotics, as well as for rational design of new orally absorbed antibiotics.

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