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ROLE OF γ-GLUTAMYLTRANSFERASE IN PUTRESCINE UPTAKE BY RAT TYPE II PNEUMOCYTES

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Abstract—Putrescine uptake in type II pneumocytes is a carrier-mediated active process. Our hypothesis was that oligoamines might be taken up into the cell at least in part by γ-glutamyltransferase (γ-GT). This was investigated in rat type II pneumocytes 24 hr after their isolation. Preexposure to 125 μM L-buthionine-[SR]-sulfoximine (BSO) or 100 μM diethylmaleate (DEM), both of which affect intracellular glutathione (GSH) only, were found to decrease GSH by 85% ($p < 0.05$) and 62%, respectively ($p < 0.05$), without change in [^{3}H]-putrescine uptake. Preexposure to 20 μM N-ethylmaleimide (NEM), which affects intra- and extracellular GSH, decreased intracellular GSH by 79% ($p = 0.015$) and putrescine uptake by 39% ($p = 0.03$). Selective extracellular GSH depletion by 10 μM copper-o-phenanthroline complex (CuP) led to a decrease in putrescine uptake of 41% ($p = 0.001$), while intracellular GSH remained unchanged. Specific inhibition of γ-GT by 5–20 mM serine-borate or 5 mM acivicin gave similar degrees of putrescine uptake inhibition (39.5% and 40.5%). The kinetic properties of the putrescine uptake system in the presence of acivicin and serine-borate indicated that the V_{max} decreased by 25%, while K_m remained unchanged. In experiments with pure γ-GT, the oligoamines putrescine, spermidine and spermine, and cystamine proved to be acceptor substrates for γ-GT, all having similar efficiencies (V_{max}/K_m); methylglyoxal-bis-(guanyl-hydrazone) and paraquat were not accepted. As extracellular GSH is required for γ-GT, and because its extracellular depletion inhibits putrescine uptake as much as specific inhibition of γ-GT, we suggest that 30–40% of the putrescine uptake in type II pneumocytes occurs by γ-GT and that, therefore, at least two systems are involved in the uptake of putrescine.

Key words: putrescine; γ-glutamyltransferase; paraquat; cystamine; polyamines; cell culture

The oligoamines (or polyamines) putrescine, spermidine, and spermine are organic cations that play an important role in cell growth, differentiation, and proliferation [1]. Previous studies have shown that one or more transport systems might be involved in the cellular uptake of oligoamines, and that pulmonary type II pneumocytes especially exhibit high uptake activity [2–5]. The pneumotoxic herbicide paraquat has been shown to accumulate in lung cells by a process that is apparently identical to that which accumulates putrescine [5, 6]. Although many authors have studied the characteristics of the paraquat/polyamine uptake system(s) [2–7], the transport system itself has not yet been identified in mammalian cells.

Previous investigations have indicated that –SH groups are required for oligoamine uptake [7–9]. Glutathione (GSH) is the most abundant intracellular thiol, and has many biological functions. It has been proposed by Meister and co-workers [10] that circulating GSH, produced principally by the liver, is the major source of the cysteine required for *de novo* intracellular GSH synthesis through the sequential actions of γ-glutamyltransferase (γ-GT), γ-glutamylcysteine synthetase, 5-oxoprolinase, and glutathione synthetase (Fig. 1). γ-GT, which is membrane-bound and localized on the external surface of cells, is involved in the transport of amino acids across the cell membrane into the cell [11–13]. As a first step in the γ-GT cycle, γ-GT breaks the γ-glutamylcysteine bond of extracellular GSH, and the γ-glutamyl moiety is transferred to an acceptor substrate, usually an amino acid, thus resulting in the formation of a γ-glutamyl-dipeptide. Subsequently, the γ-glutamyl-dipeptide, as well as cysteinylglycine, is taken up by the cell and catabolized to glutamate and free amino acids by the action of γ-glutamylcyclotransferase and cysteinylglycine dipeptidase. It has been shown that certain tumor cells express more γ-GT on their cell membranes compared to normal cells, and this might be correlated with an unfavourable prognosis [14, 15].

Since both the putrescine uptake system and, at least indirectly, the γ-glutamyl cycle are involved in growth and differentiation of cells, our hypothesis is that one of the routes by which oligoamines are taken up into the cell is via the γ-glutamyl cycle. Binding of the amino group to the carboxamide group of the γ-glutamyl residue might be possible by analogy to the transglutaminase-catalysed binding of diamines and polyamines to peptide chains [16, 17]. Indeed, γ-GT not only binds to amino acids, dipeptides, and tripeptides, but studies on γ-GT in plants have shown that amines may also function as acceptor substrates for this "high capacity-low specificity" enzyme [18].

The aim of our study was to verify (a) whether γ-GT is involved in the uptake of putrescine; and (b) whether manipulation of GSH level will modify the uptake of putrescine.

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† Abbreviations: BSO, L-buthionine-[SR]-sulfoximine; DEM, diethylmaleate; NEM, N-ethylmaleimide; CuP, copper-o-phenanthroline; MGBG, methyl-glyoxal-bis-(guanyl-hydrazone); BSA, bovine serum albumin; γ-GT, γ-glutamyltransferase; GSH, glutathione; DTNB, 5,5-dithio-(2-nitro)-benzoic acid; FCS, fetal calf serum; SSA, 5-sulfosalicylic acid; PBS^+ and PBS^-, phosphate buffered saline with or without calcium and magnesium, respectively; V_{max}, the maximal rate of uptake; K_m, the medium concentration at which the rate of uptake is half V_{max}; dpm, disintegrations per minute; cpm, counts per minute.

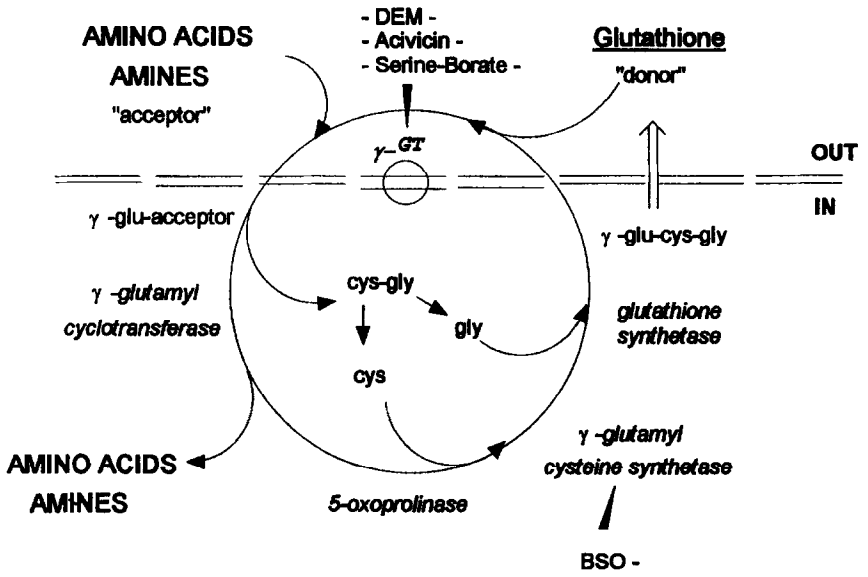

Fig. 1. Schematic representation of the γ-glutamyl cycle. Amino acids are translocated by γ-GT across the cell membrane by binding to the γ-glutamyl moiety of GSH, and are released by the action of γ-glutamyl cyclotransferase within the cell. The long arrowheads indicate sites of inhibition (after Meister *et al.* [29]). γ-GT = γ-Glutamyltransferase, DEM = diethylmaleate, BSO = L-buthionine-[SR]-sulfoximine.

MATERIALS AND METHODS

Animals and materials

Male Wistar rats (140–170 g) were obtained from an in-house strain. [1,4(n)-^{3}H]putrescine dihydrochloride [14.6 Ci/mmol (1 μCi/μl)] was purchased from Amersham International Ltd. (Brussels, Belgium), and Emulsifier Safe scintillant and plastic scintillation vials (5 ml) were purchased from Packard N.V. (Zellik, Belgium). Paraquat dichloride (99% pure, Plant Protection Division, ICI plc, now Zeneca) was a gift from Dr L. L. Smith (Central Toxicology Laboratory, ICI plc, now Zeneca). Putrescine dihydrochloride, L-serine, sodium tetraborate, acivicin (L-(αS,5S)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid), MGBG†, cystamine, L-cysteine, glutathione, NEM, DEM, BSO, cupric sulfate, o-phenanthroline, L-γ-glutamyl-p-nitroanilide, glycylglycine, spermidine, spermine, proline, trypsin type I (EC 3.4.214), trizma base (Tris), bovine serum albumine (BSA), DNAse I, glutathione reductase type IV (EC 1.6.4.2, 100 U/mL), γ-glutamyl-transferase type I (EC 2.3.2.2., 6 U/mg), β-Nicotinamide-adenine-dinucleotide-phosphate, reduced form (β-NADPH), Percoll, and 5,5-dithio-(2-nitro)-benzoic acid (DTNB) were all purchased from Sigma Germany (Filter Service NV/SA, Eupen, Belgium). Waymouth's 752/1 medium, Dulbecco's Modified Eagle 11880 Medium without phenol red and GSH (DMEM), fungizone (amphotericin 250 μg/mL), L-glutamine (200 mM), penicillin-streptomycin solution (10,000 U–10,000 μg/mL, respectively), Hepes, and fetal calf serum (FCS) were purchased from Gibco (Merelbeke, Belgium), and 5-sulfosalicylic acid (SSA) was purchased from Merck, Germany. Protein assay dye solution was purchased from Bio-Rad (Brussels, Belgium). All other chemicals were purchased from U.C.B. Belgium (Vel NV/SA, Leuven, Belgium). Ninety-six-well cell-culture plates coated with extracellular matrix (ECM) were purchased from Biological Industries, Glasgow, U.K.

Isolation of rat type II pneumocytes

The isolation procedure was based on the methods of Richards *et al.* [19] and Hoet *et al.* [3]. Briefly, rats were deeply anaesthetized by an intraperitoneal injection of 60 mg pentobarbital/kg body weight (Nembutal, 60 mg/ml). After perfusion of the lungs with 0.9% NaCl, the lungs and trachea were removed from the thoracic cavity, and 6–8 mL 0.9% NaCl were instilled and withdrawn five times via a tracheal cannula. After the last lavage, the lungs were filled with a trypsin solution (500 mg trypsin in 200 mL PBS$^+$ containing 130 mM NaCl, 5.4 mM KCl, 11 mM glucose, 10.6 mM Hepes, and 2.6 mM Na_2HPO_4 adjusted to pH 7.4, plus 1.9 mM $CaCl_2$ and 1.29 mM $MgSO_4$). After a first incubation with trypsin for 5 minutes, fresh trypsin solution was instilled for 30 min at 37°C. After this digestion period, the trachea and main bronchi were removed from the lungs; the lungs were chopped for 5 minutes with scissors; 5 ml FCS and 15 ml DNase solution (250 μg/ml PBS$^-$) were added; and the tube was shaken by hand for 5 minutes. The digest was filtered through a cotton gauze and two Nylon filters (mesh 80 and 25 μm; Heleine Cavenaile PVBA/SPRL, Brussels, Belgium), and centrifuged at 250 g for 20 minutes (4°C) on a discontinuous Percoll gradient (density 1.089 g/mL and 1.040 g/mL). The creamy layer above the heavy gradient was collected, rinsed in PBS$^-$, suspended in Waymouth's medium supplemented with DNase 50 μg/mL, 2% FCS, 2% fungizone, and 2% penicillin-streptomycin solution, and incubated for 1 hr in a Petri dish placed in a CO_2 incubator (10% CO_2, 37°C) in order to let the macrophages attach. The unattached cells were then spun down (250 g, 10 min) and resuspended in the final Waymouth's medium containing 10% (v/v) FCS, 1% fungizone, 1% penicillin-streptomycin solution, and 1% L-glutamine. The cells were counted in a Buerker chamber and plated on 96-well plates (100,000 type II cells/200 μl/well). The cells were used 24 hr after plating, and were rinsed with PBS$^+$

to remove non-adherent cells. This procedure yielded more than 95% viable type II pneumocytes, as determined by Trypan Blue exclusion.

GSH determination

Total GSH (GSH + GSSG) was determined using GSH-reductase according to the procedure of Anderson [20], by which GSH can be detected in the picomolar range. 700 μL buffer (143 mM sodium phosphate, 6.3 mM Na_4-EDTA, and 0.35 mM β-NADPH, pH 7.5), 100 μL 6 mM DTNB, 150 μl water, and 50 μL sample containing 2.5% SSA (w/v) were transferred to a cuvette and heated to 37°C. Then 10 μL GSH-reductase (100 U/mL) were added, with mixing, to initiate the assay. The change in absorbance was measured at 412 nm, using a Beckman DU65 spectrophotometer. For each run, a standard curve was prepared. The samples were obtained by treating the cells with 50 μl 2.5% SSA (w/v) per well for 30 min. For each data point, the mean GSH concentration of two wells was taken. The protein concentration for each well (±50 μg) was determined afterwards by dissolving the precipitated proteins in 200 μL NaOH and, after neutralization with 1 M HCl, the concentration was measured using the method of Bradford [21]. The GSH concentration was expressed as nmol/mg protein. GSSG was not determined because it was not playing a role in this study. Preliminary experiments (data not shown) indicated that prior detachment of the cells from the culture plate by trypsin, followed by precipitation in SSA, centrifugation, and GSH determination in the supernatant gave similar results, but because this method was cumbersome, it was abandoned. For the measurement of GSH in the extracellular fluid, the Waymouth's medium was replaced by DMEM (i.e. GSH free medium) after the cells had been rinsed with PBS^+.

Accumulation of putrescine in cultured type II pneumocytes

Putrescine uptake experiments were performed according to Hoet *et al.* [3]. Briefly, after removal of the Waymouth's medium, the cells were incubated at 37°C or 4°C for 1 hr in 200 μL PBS^+ containing 10 μM putrescine and 0.1 μCi [^{3}H]-putrescine. After incubation, the wells were rinsed three times with 200 μL cold PBS^+ containing 10 μM unlabelled putrescine. Next, the cells were dissolved in 200 μl NaOH (0.1 M) for at least 3 hr and 4.5 mL Emulsifier scintillant were added, and radioactivity was measured by liquid scintillation spectrophotometry, using a Beckman ls5000ce counter, fitted with appropriate quench correction curves to convert cpm to dpm. The accumulation of putrescine, expressed as pmol/mg protein/hr, was obtained from the tissue-associated radioactivity, the specific activity of [^{3}H]-putrescine, and the protein concentration of identically treated cells, measured as above after neutralisation with 0.1 M HCl prior to protein measurement.

Intra- and extracellular GSH and putrescine uptake

Prior to putrescine uptake studies, cells were preincubated at 37°C with four different GSH-modifying compounds [12]: 125 μM BSO in Waymouth's medium for 2 hr; 100 μM DEM (prepared from a 50 mM DEM stock solution made up of 86 μL DEM dissolved in 0.3 mL ethanol and 9.7 mL PBS^+) in Waymouth's medium for 2 hr; and 20 μM NEM in PBS^+ for 5 min. Concomitant with the putrescine uptake, the cells were also exposed to a 10 μM copper-o-phenanthroline (CuP) complex, with a 1:2 molar ratio of Cu:P (prepared from a 10 mM CuP stock solution, made up by mixing 24.9 mg cupric sulphate dissolved in 9.7 mL water with 39.6 mg phenanthroline dissolved in 0.3 mL ethanol), in PBS^+ [22, 23]. Controls were also exposed to ethanol, as appropriate.

Putrescine uptake inhibition

Concomitant with the putrescine uptake, cells were exposed to possible modulators of γ-GT: 5, 50, 500, or 5000 μM acivicin; 5 mM L-serine alone, 20 mM sodium tetraborate alone, 2.5 mM L-serine plus 10.0 mM sodium tetraborate, or 5.0 mM L-serine plus 20.0 mM sodium tetraborate, all in PBS^+, adjusted for pH and osmolarity; 10 or 20 μM GSH; 10 or 20 μM L-cysteine; 20 or 40 μM MGBG; 12.5, 25, 50 or 100 μM cystamine; 100, 200, 400, 1250, or 2500 μM paraquat.

The γ-GT assay

γ-GT was assayed as described by Meister *et al.* [10]. Briefly, 0.2 mL L-γ-glutamyl-p-nitroanilide (final concentration 1 mM), 0.59 mL 0.1 M Tris-HCl buffer (pH 8.0), and 0.2 mL of the acceptor substrate were brought to 37°C in a cuvette; 10 μL of γ-glutamyltransferase type I (60 units/mL) were added, the cuvette shaken, and the change in absorbance recorded at 410 nm for 2 min using a Beckman DU65 spectrophotometer. Enzyme activity was expressed as micromoles p-nitroaniline formed per minute (Units), using a molar extinction coefficient of 568 $mM^{-1}cm^{-1}l^{-1}$ for the conversion from ΔA to Units/L [24]. The relatively high donor substrate concentration was used to minimize hydrolysis [9]. The assay was performed with 0–160 mM of different potential acceptors: glycylglycine, L-cysteine, putrescine, spermidine, spermine, MGBG, cystamine, paraquat, and cystamine. After subtracting the change in absorbance due to autotranspeptidation, K_m and V_{max} were calculated. The Michaelis-Menten hyperbola of L-cysteine was compared with that in the presence of 2.5 and 10 mM Ca^{2+}, and 10 mM putrescine. In addition, the known γ-GT inhibitors acivicin and serine-borate were tested.

Viability of cultured type II cells

Viability and cellular integrity were verified by Trypan Blue exclusion each time the cells were treated with the various test reagents.

Analysis of data

All experiments were performed on cells obtained from at least three animals with approximately 15×10^6 cells/animal, which were divided, per animal, into control and treatment groups. All results were obtained from separate experiments and expressed as means ± SD. K_m and V_{max} were calculated by a computerized curveplotting procedure (Fig.P software) or Hane's plot [25]. Statistical analysis was performed by the paired *t*-test using the SAS/STAT package (6th version). The level of significance was set at $P < 0.05$.

RESULTS

Intra- and extracellular GSH and putrescine uptake

GSH levels were manipulated by four different GSH modifying compounds.

1. After 2 hr preincubation with 125 μM BSO, an irreversible inhibitor of γ-glutamyl-synthetase [12], in-

tracellular GSH decreased from 5.7 ± 0.4 nmol/mg at t = 0 hr to 0.9 ± 0.5 nmol/mg at t = 4 hr (P = 0.008), while extracellular GSH was unchanged. Putrescine uptake was not different from the control values (Fig. 2A). The apparent increase in putrescine uptake at t = 0 hr could not be confirmed by further experiments, unlike the increase in GSH, which was reproducible (data not shown).

2. After 2 hr preincubation with 100 μM DEM, which selectively depletes cytoplasmic and nuclear GSH while conserving extracellular and mitochondrial GSH [12], intracellular GSH decreased from 5.0 ± 0.3 nmol/mg at t = −2 hr, to 1.9 ± 0.3 nmol/mg at t = 0 hr (P = 0.005), again with no change in putrescine uptake (Fig. 2B).

3. To decrease intra- and extracellular GSH, cells were preincubated with 20 μM NEM for 5 min [22]. Intracellular GSH decreased from 3.8 ± 0.1 nmol/mg at t = −2 hr to 0.8 ± 0.3 nmol/mg at t = 1 hr (P = 0.015), while putrescine uptake decreased by 39 ± 12% (P = 0.03) (Fig. 2C).

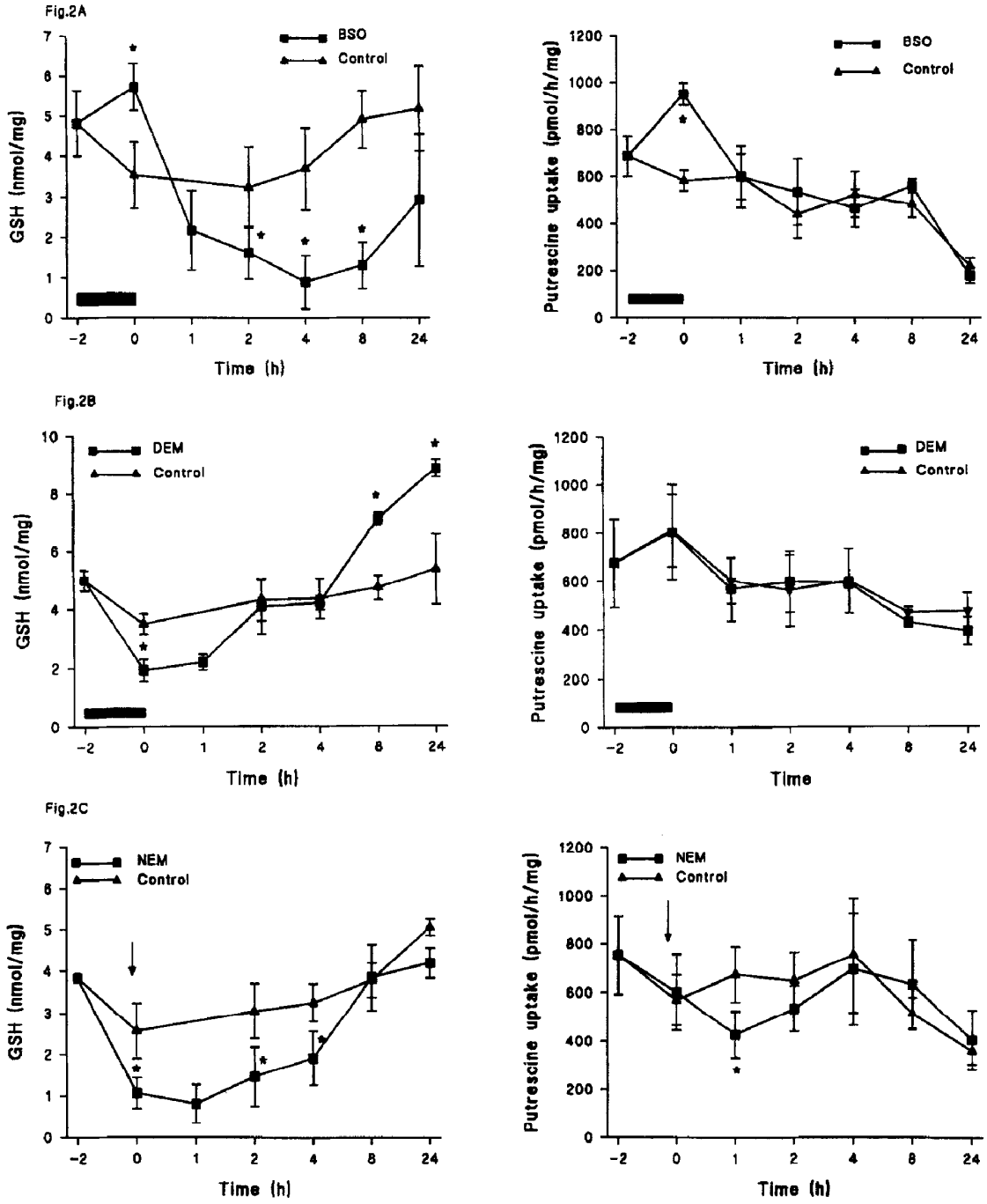

Fig. 2. The relationship between intracellular GSH and putrescine uptake by rat type II pneumocytes: (A) 2 hr preincubation with 125 μM BSO (horizontal bar); (B) 2 hr preincubation with 100 μM DEM (horizontal bar); (C) 5 min preincubation with 20 μM NEM (arrow); and (D) 1 hr coincubation with 10 μM copper-o-phenantroline complex (CuP). Data ± SD, n = 3, $^*p < 0.05$ vs control (paired t-test).

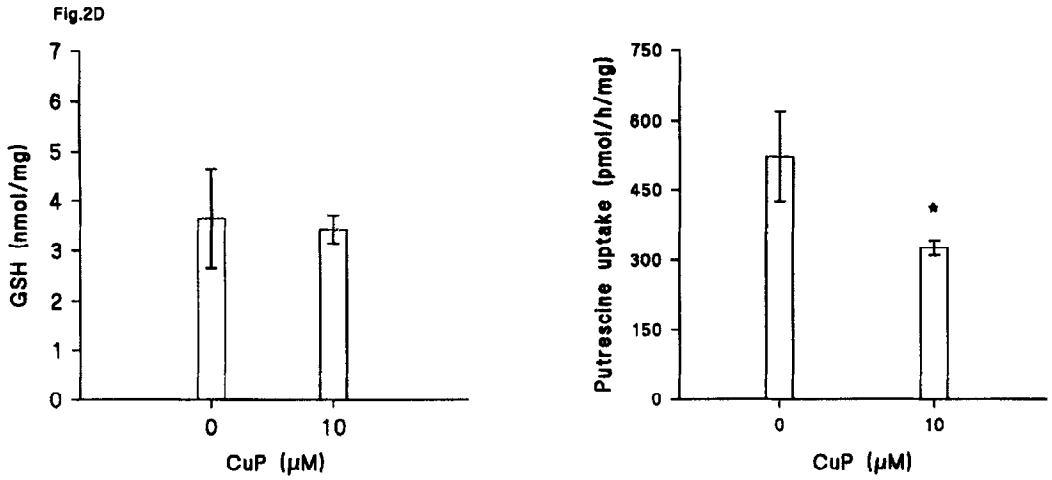

Fig. 2. Continued

4. Selective extracellular GSH depletion was achieved by incubation with a low concentration of the CuP complex [22]. The 10 μM CuP solution did not influence intracellular GSH during 1 hr incubation (3.7 ± 0.8 nmol/mg before vs 3.4 ± 0.2 nmol/mg after incubation), but putrescine uptake decreased by 41 ± 7% ($P = 0.001$). Extracellular GSH was not detectable after incubation with CuP (control values 0.1–0.4 mM) (Fig. 2D).

Putrescine uptake inhibition (Table 1)

Reversible inhibition of γ-GT by 5–20 mM serine-borate [10] resulted in a decrease in putrescine uptake of 39.5 ± 5.7% ($n = 42$, $P < 0.001$). Five mM L-serine, 20 mM borate, or 2.5–5 mM serine-borate gave no inhibition of putrescine uptake, but 5.0–10 mM serine-borate led to 32.2 ± 3.2% inhibition. The specific and irrevers-

Table 1. The effect of different inhibitors of [^{3}H]putrescine uptake in rat type II pneumocytes

Inhibitor		n	% Decrease in uptake I	% Decrease in uptake I + SB
L-Serine	5 mM	6	6.9 ± 8.0	NA
Borate	20 mM	6	8.5 ± 7.1	NA
SB	2.5–5 mM	3	−0.1 ± 0.4	NA
SB	5–10 mM	6	32.2 ± 3.8*	NA
SB	5–20 mM	42	39.5 ± 5.7*	NA
Acivicin	5 μM	3	11.9 ± 4.4	ND
	50 μM	3	14.8 ± 12.0	ND
	500 μM	3	32.4 ± 6.0*	ND
	5 mM	6	40.5 ± 5.0*	40.0 ± 7.0
Glutathione	10 μM	3	20.1 ± 17.0	44.0 ± 10.1†
	20 μM	3	49.0 ± 9.0*	44.0 ± 10.0
Glygly	10 μM	3	9.4 ± 7.2	36.0 ± 5.2†
	20 μM	3	7.5 ± 6.7	36.0 ± 4.3†
L-Cysteine	10 μM	3	14.0 ± 8.0	34.3 ± 11.9†
	20 μM	3	18.6 ± 12.5	37.3 ± 13.6†
MGBG	20 μM	6	56.9 ± 18.3*	83.0 ± 5.9†
	40 μM	3	62.0 ± 0.1*	ND
Cystamine	12.5 μM	3	41.6 ± 6.0*	58.2 ± 8.6
	25 μM	3	55.0 ± 11.0*	66.9 ± 13.7
	50 μM	3	64.3 ± 4.7*	77.2 ± 15.1†
	100 μM	3	78.4 ± 3.6*	90.6 ± 3.1†
Paraquat	100 μM	3	8.2 ± 11.5	44.0 ± 8.6†
	200 μM	3	12.9 ± 7.0	45.7 ± 8.4†
	400 μM	3	23.9 ± 6.0*	52.4 ± 8.7†
	1250 μM	3	39.7 ± 13.1*	67.3 ± 5.0†
	2500 μM	3	43.0 ± 12.0*	70.0 ± 3.3†

Inhibition of [^{3}H]putrescine uptake in freshly isolated rat type II pneumocytes, 24 hr after their isolation. NA not applicable, ND not determined, I = Inhibitor, SB = L-Serine-Borate (5–20 mM), I + SB = Inhibitor plus L-Serine-Borate, MGBG = methylglyoxal-bis-(guanyl-hydrazone), Glygly = Glycylglycine. I* $P < 0.05$ compared to the control values, I + SB; †$P < 0.05$ compared to I (paired t-test), means ± SD.

ible γ-GT inhibitor acivicin [10] reduced putrescine uptake in a dose-dependent fashion, with a maximal inhibitory effect reached at 5 mM. At this concentration, putrescine uptake was decreased by 40.5 ± 5% ($P = 0.005$). The combination of both inhibitors did not augment inhibition. Cell viability was verified by Trypan Blue exclusion, but no signs of toxicity could be detected.

Ten and 20 μM GSH inhibited putrescine uptake by 20.1 and 49.0%, respectively, without additional inhibition by serine-borate for the highest GSH concentration. L-cysteine (10 and 20 μM), glycylglycine (10 and 20 μM) and L-serine (5 mM) did not inhibit putrescine uptake. Preliminary experiments (data not shown) indicated that the K_i (concentration at which K_m is doubled) for MGBG was 0.57 μM; at concentrations of 30 and 60 times the K_i, uptake was inhibited by 56.9 ± 18.3% and 62.0 ± 0.1%, respectively ($P < 0.05$); paraquat, at the highest concentration, inhibited the uptake by 43.0 ± 12.0% ($P < 0.05$). MGBG or paraquat plus serine-borate resulted in an additive inhibitory effect, approaching the sum of the inhibitions caused by either compound alone. Preliminary experiments (data not shown) indicated that the K_i for cystamine was 9.2 μM; at 10 times the K_i, cystamine inhibited putrescine uptake by approximately 75%, with only a minor additive inhibitory effect of serine-borate.

The apparent K_m for putrescine uptake was 7.4 ± 1.9 μM with a V_{max} of 1030 ± 76 pmol/mg/hr (Fig. 3). After addition of serine-borate, the K_m remained unchanged (7.3 + 2.2 μM), but the V_{max} decreased by 25% (773 ± 67 pmol/mg/hr, $p < 0.01$). The same results were obtained after addition of acivicin (5 mM): The K_m was 5.9 ± 1.4 μM, and the V_{max} decreased to 757 ± 47 pmol/mg/hr, $P < 0.01$). The combination of acivicin and serine-borate resulted in a K_m of 3.6 ± 1.7 μM ($p < 0.05$, and a V_{max} of 734 ± 130 pmol/mg/hr ($P < 0.01$). However, if a Hane's plot was used to calculate the K_m and V_{max} for the inhibition with acivicin and serine-borate together, the same values as for the inhibition with serine-borate or acivicin alone were obtained.

The γ-GT assay (Table 2)

The assay was validated with glycylglycine (K_m 5.0 mM) and with proline, which as expected [13] was negative. Serine-borate and acivicin inhibited γ-GT by 90% and 85%, respectively. Table 2 shows that putrescine, spermidine, and spermine can serve as acceptor substrates for γ-GT, giving about equal K_m values and V_{max}/K_m ratios. Using the latter index as a measure of the relative efficiency of the substrate to serve as acceptor, it appears that the acceptor substrates glycylglycine and L-cysteine are approximately 30 and 10 times, respectively, more efficient than putrescine. The diamine cystamine also acts as an acceptor substrate for γ-GT, with

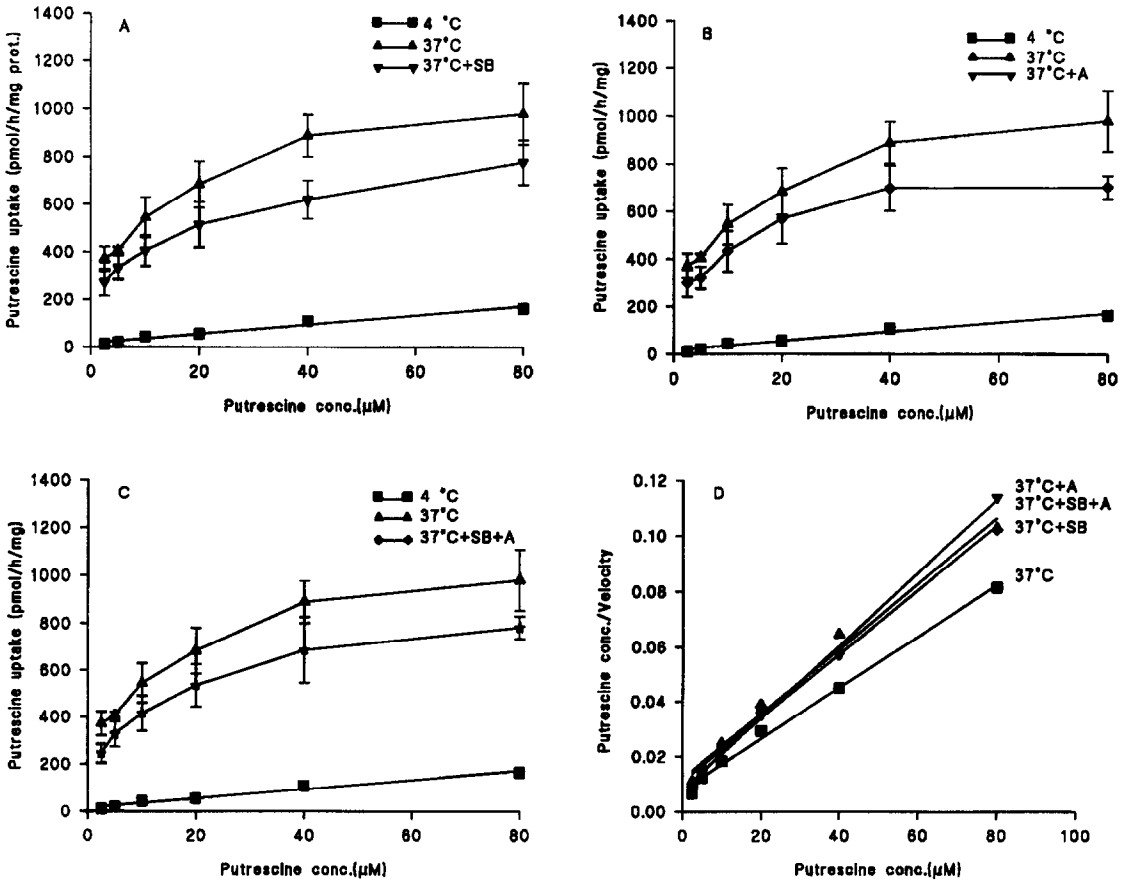

Fig. 3. The non-competitive inhibition of putrescine uptake by acivicin and serine-borate in rat type II cells. Abbreviations: SB = serine-borate; A = acivicin. Panel D: Hane's plot: K_m remains the same. V_{max} decreases by 25% during inhibition with SB, A or SB + A. Means ± SD, $n = 3$ (paired t-test). Units of y-axis were taken as ratio of putrescine concentration and values of y-axis of panels A, B, or C.

Table 2. Kinetic properties of γ-GT for different potential acceptors

Acceptor	K_m (mM)	V_{max} (U/L)	V_{max}/K_m min^{-1}
Glygly	5.0 ± 1.9	2167 ± 384	433
L-Cysteine	4.5 ± 2.5	589 ± 230	131
Cystamine	94.6 ± 10.1	370 ± 14	4
Putrescine	4.2 ± 1.0	55 ± 4	13
Spermidine	1.1 ± 0.4	15 ± 1	14
Spermine	4.2 ± 0.7	18 ± 11	4
Paraquat	no acceptor		
MGBG	no acceptor		
Proline	no acceptor		

Glygly = glycylglycine, U = units (i.e. micromoles nitroaniline release/min). Experimental conditions: 0.2 mL L-γ-glutamyl-p-nitroanilide 1 mM, 0.59 ml 0.1 M Tris-HCl buffer pH 8.0, 0.2 mL acceptor, and 10 μL γ-GT (60 U/mL) at 37°C. The absorbance was measured at 410 nm for 2 min (means of three observations ± SD).

high K_m and V_{max} values, but similar enzyme efficiency compared to the oligoamines. Although paraquat and MGBG [2, 3, 5, 6] are known inhibitors of (one of) the putrescine uptake pathway(s), they proved not to be acceptor substrates for γ-GT.

As putrescine has been described as an "organic calcium" because of its two positive charges at physiological pH, and because monovalent cations and divalent metal ions are reported to activate γ-GT [10], we investigated the effect of 2.5 and 10 mM Ca^{2+}, and 10 mM putrescine on the K_m and V_{max} of L-cysteine (Fig. 4). The apparent kinetic properties for L-cysteine, L-cysteine plus 2.5 mM Ca^{2+}, L-cysteine plus 10 mM Ca^{2+}, or L-cysteine plus 10 mM putrescine were unchanged with values of 4.5, 3.4, 4.3, and 4.3 mM, respectively, for K_m, and 589, 527, 582, and 592 U/L for V_{max}.

DISCUSSION

The oligoamines putrescine, spermidine, and spermine are required for cell growth, differentiation, and proliferation. It has been established that tumors or rapidly proliferating cells require oligoamines in increasing amounts [1]. Especially in these situations, oligoamine uptake can complement or substitute for *de novo* biosynthesis of oligoamines. This uptake has been studied in a number of cells, and was observed to be a saturable, carrier-mediated, and energy-dependent system [2–9]. Many studies of this system have focused on its use by other compounds, as evidenced by the competitive inhibition of polyamine uptake by their analogues or vice versa, but the transporter has not yet been identified or purified [6]. Genes responsible for the phenotypic expression of oligoamine transport have been cloned from both *E. coli* and human DNA [26, 27], and a number of different clones have been identified with different properties, which might be consistent with the multiplicity of the polyamine transport system. However, the amino acid sequences of the transport protein(s) are as yet unknown. Mainly on the grounds of kinetic considerations, some authors believe that only one uptake system is involved [3], whereas others have suggested at least two uptake systems [2, 6, 8], some of them Na^+ dependent and sensitive to SH-group reagents [28].

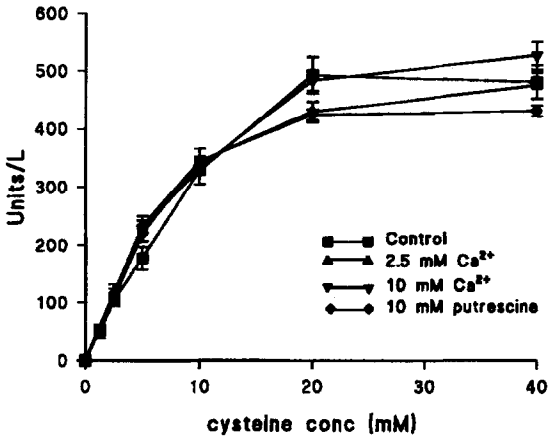

Fig. 4. Effect of 2.5 or 10 mM Ca^{2+}, or 10 mM putrescine on the activity of γ-GT; 0.2 mL L-γ-glutamyl-p-nitroanilide 1 mM, 0.59 mL 0.1 M Tris-HCl buffer pH 8.0, 0.2 mL L-cysteine at different concentrations, and 10 μL γ-GT (60 U/mL) were mixed, and the change in absorbance measured at 410 nm for 2 min at 37°C. There was no difference between the K_m and V_{max} values of the different curves (means ± SD).

It is assumed that GSH and closely related γ-glutamyl compounds are the physiological substrates of the enzyme γ-GT [10]. Because γ-GT and its substrate GSH are located on opposite sides of a membrane, intracellular GSH may be first exported to the extracellular space, where it is bound to γ-GT [29] (Fig. 1).

Summary of findings and hypothesis

Our *hypothesis* was that one of the routes by which the oligoamine putrescine might enter the type II pneumocyte is via γ-GT. Our findings can be *summarized* as follows: exposure of type II pneumocytes to BSO and DEM, both of which decrease only intracellular GSH [12], did not change putrescine uptake, but exposure to NEM, which affects intra- and extracellular GSH [12, 22], decreased putrescine uptake. Selective extracellular GSH depletion with CuP, while conserving intracellular GSH [22], decreased putrescine uptake as well. Specific inhibition of γ-GT by serine-borate gave the same degree of putrescine uptake inhibition as that caused by acivicin and CuP. L-serine, cysteine, and glycylglycine did not inhibit putrescine uptake, but GSH did, although without any additive inhibitory effect of serine-borate. MGBG and paraquat decreased putrescine uptake, but even though "infinite" concentrations (compared to the K_i values) were used, putrescine uptake could be inhibited only by 62 and 43%, respectively. If serine-borate was added, the inhibition approached the sum of the inhibition by MGBG or paraquat and serine-borate. Cystamine inhibited putrescine uptake by approximately 75%, with only 15% additional inhibitory effect of serine-borate being obtained at the highest concentrations of cystamine. The characteristics of the putrescine uptake system in the presence of acivicin and serine-borate show that V_{max} decreased by 25% with no change in K_m. In test-tube experiments with pure γ-GT, the oligoamines putrescine, spermidine and spermine, and cystamine proved to be acceptor substrates for γ-GT, all having similar efficiencies (V_{max}/K_m), but MGBG and paraquat were not accepted as substrates.

Technical aspects

L-serine in the presence of borate is known to inhibit γ-GT in a reversible way [10]. Neither compound alone

inhibits the enzyme, which means that effects not dependent on the simultaneous presence of both compounds may not be attributed to inhibition of γ-GT [10]. In our experiments, L-serine or borate alone did not inhibit putrescine uptake, making the possibility of atypical effects (i.e. changes in charge) less likely. Moreover, in the γ-GT assay the kinetic parameters did not change in the presence of calcium, or the dicationic putrescine (Fig. 4), and it was possible to inhibit putrescine uptake as much, and with the same kinetic properties, by acivicin, a well-established irreversible γ-GT inhibitor, by virtue of its covalent binding to the glutamyl portion of the substrate binding site [10]. The aim of the γ-GT study was to demonstrate that oligoamines can serve as acceptor substrate for the enzyme, and to express the efficiency of the enzyme to handle oligoamines compared to other known acceptor substrates. To validate our experimental setup, we determined the K_m for glycylglycine, with the value found (5.0 mM) being in the same range as those found by others (2.96 and 9.5 mM) [10, 24].

Interpretation

Our results indicate that extracellular –SH groups are required for putrescine uptake. These –SH groups might belong to membrane proteins, to the transporter protein, or to the donor substrate for γ-GT (i.e. GSH). If extracellular GSH was depleted by CuP, putrescine uptake was inhibited as much as by the specific γ-GT inhibitors serine-borate and acivicin. These results support our hypothesis that putrescine can be taken up via the γ-glutamyl cycle. Presuming that the γ-GT route is completely inhibited by serine-borate or acivicin, the fact that similar K_m values were found pre- and post-inhibition might be explained by equal affinities of putrescine for both systems. This would be consistent with our previous kinetic studies on putrescine uptake in type II pneumocytes [3].

The observations that putrescine uptake inhibition by serine-borate was additive to MGBG and paraquat (Table 2), and that the oligoamines proved to be acceptor substrates for γ-GT in the γ-GT assay, in contrast to MGBG and paraquat, indicate that there could be at least two uptake pathways. Oreffo *et al.* [30] came to the same conclusion: "... we cannot exclude the possibility that type II cells have two separate uptake systems, one involving competitive inhibition of the diamine (paraquat) by the polyamine (putrescine), and the other solely for polyamine accumulation." Data from our experiments with cystamine suggest that cystamine has affinity for both pathways, an observation shared by others [31]. There is likely a higher inhibitory affinity for the γ-GT pathway, because serine-borate had only a 15% additive inhibitory effect.

Specificity studies [10] have provided evidence for three separate subsites in the active centre of γ-GT: the γ-glutamyl donor site, with broad optical and steric specificity, and the acceptor site, consisting of subsites 2 and 3 for the cysteinyl and glycyl moieties of GSH. Acceptor amino acids will only produce an inhibition of γ-GT if they bind to the acceptor binding site 2 or 3, if they overlap with a portion of the γ-glutamyl donor site, or if they bind to the sites that normally bind the amino and carboxyl groups of the γ-glutamyl donor [16]. Dipeptides (glycylglycine) seem to bind only to the acceptor subsite (subsite 2 or 3) [32, 33]. The fact that L-serine, cysteine, and glycylglycine did not inhibit putrescine uptake might indicate that oligoamines do not bind to the acceptor binding site, but probably in part to the γ-glutamyl donor site. The inhibition by GSH might be explained by competitive inhibition with the oligoamines at this γ-glutamyl donor site. A free terminal amino group is probably also a prerequisite, since MGBG and paraquat do not appear to be acceptor substrates for γ-GT.

CONCLUSION

Our data indicate that 30–40% of putrescine uptake in rat type II pneumocytes may occur via γ-GT, and that at least two systems are thus involved in putrescine uptake. Moreover, there is evidence for a link between extracellular GSH and putrescine uptake. These results might be of clinical relevance: As putrescine is involved in growth and differentiation of cells, changes in extracellular GSH or γ-GT by, for example, oxidative stress might have repercussions on normal cell growth and differentiation.

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