Expression and protein kinase C-dependent regulation of peptide/H⁺ co-transport system in the Caco-2 human colon carcinoma cell line

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The characteristics of the transport of the dipeptide glycylsarcosine were studied in the human colon carcinoma cell line Caco-2 grown as a monolayer on impermeable plastic support. Transport of glycylsarcosine in these cells was found to be Na⁺-independent, but was stimulated by an inwardly directed H⁺ gradient. This H⁺-dependent transport of glycylsarcosine was inhibited by di- and tri-peptides and also by the β -lactam antibiotic cephalixin, but was unaffected by the amino acids glycine and leucine. The transport system exhibited a Michaelis–Menten constant (K_m) of 1.1 ± 0.1 mM for glycylsarcosine. The specific activity of the transport system in this cell line was found to be maximal when the cultures were confluent. Treatment of the cells with phorbol esters which activate protein kinase C resulted in a significant inhibition of the transport system. This

inhibition was specific and could be blocked if treatment was done in the presence of staurosporine, an inhibitor of protein kinase C. Kinetic analysis revealed that the inhibition was associated with a decrease in the maximal velocity, the K_m remaining unaffected. The phorbol-ester-induced inhibition of the peptide-transport system was not prevented by co-treatment with cycloheximide, an inhibitor of cellular protein synthesis. In addition, there was no change in the intracellular pH following treatment with the phorbol ester, suggesting that the effect was not due to alterations in the transmembrane pH gradient. It is concluded that the peptide/H⁺ co-transport system, which is known to exist in the normal intestine, is expressed in Caco-2 cells and that the function of the transport system is under the regulatory control of protein kinase C.

INTRODUCTION

Among the various transport systems available for absorption of nutrients in the small intestine, the peptide-transport system occupies a unique position because of the involvement of a protonmotive force rather than a sodiummotive force as the energy source [1–4]. This transport system accepts as substrates small peptides containing two or three amino acids. Free amino acids are excluded by the system. There is overwhelming evidence for the importance of the absorption of small peptides in the small intestine [5] and therefore the peptide-transport system that is responsible for this absorptive process plays a vital role in the maintenance of optimal protein nutrition. In addition to its natural substrates, the transport system is also capable of transporting certain pharmacological agents which possess structural features similar to those of small peptides. The peptide-transport system has been shown to be involved in the intestinal transport of pharmacologically active compounds such as β -lactam antibiotics [6–8] and bestatin [9]. The potential for nutritional, clinical and therapeutic applications of the intestinal peptide-transport system has proved to be a catalyst for the growing interest in understanding the physiological, biochemical and molecular aspects of this transport system.

The characteristics of the intestinal peptide-transport system have been investigated with different tissue preparations, including intact intestine, pieces of mucosal tissue, isolated intestinal cells and purified brush-border and basolateral membrane vesicles [5]. Recently, the human colon carcinoma cell line Caco-2 has been shown to express an Na⁺-independent peptide-transport system, as assessed by the ability of the cells to transport β -lactam antibiotics [10–12]. This cell line has been widely used as a model in intestinal transport studies [13,14]

and has the potential to be useful in investigations involving regulation of the peptide-transport system by intracellular second messengers. But, the transport system expressed in these cells has not yet been investigated using physiological substrates. In addition, to date there is no information available on the regulation of the peptide-transport system by intracellular second messengers. The purpose of the present investigation was two-fold: (i) to characterize the peptide-transport system in Caco-2 cells using the hydrolysis-resistant dipeptide glycylsarcosine [15–18], a methylated analogue of glycylglycine, as a substrate, and (ii) to determine whether the function of the transport system is modulated by protein kinase C. The results of the investigation show that glycylsarcosine is transported in these cells by an Na⁺-independent peptide-transport system whose activity is influenced by a transmembrane H⁺ gradient and that the catalytic function of this transport system is inhibited by protein kinase C. Part of this work has been presented recently at the Annual Meeting of the American Physiological Society [19].

EXPERIMENTAL

Materials

The human colon carcinoma cell line Caco-2 was obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). Minimum Essential Medium (MEM) with Earle's salts and L-glutamine, MEM non-essential amino acids, fetal-bovine serum, penicillin (10000 units/ml)-streptomycin (10000 μ g/ml) and trypsin were purchased from GIBCO-BRL (Grand Island, NY, U.S.A.). Collagen solution, unlabelled amino acids, phorbol 12-myristate 13-acetate (PMA), phorbol 12,13-dibutyrate (PDBu), mezerein, 4 α -phorbol 12,13-didecanoate (PDD), 4 α -phorbol,

Abbreviations used: MEM, minimum essential medium; DMSO, dimethyl sulphoxide; PMA, phorbol 12-myristate 13-acetate; PDBu, phorbol 12,13-dibutyrate; PDD, 4 α -phorbol 12,13-didecanoate; DMO, 5,5-dimethylloxazolidine-2,4-dione.

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staurosporine, ouabain and cycloheximide were purchased from Sigma (St. Louis, MO, U.S.A.). Unlabelled peptides were from either Sigma or Bachem Bioscience (Philadelphia, PA, U.S.A.). Cephalixin was generously given by Professor T. Hoshi, University of Shizuoka, Shizuoka, Japan.

Carrier-free $^{22}\text{NaCl}$ (sp. radioactivity 200 $\mu\text{Ci/ml}$), L-[3- ^3H]-alanine (sp. radioactivity 76.9 Ci/mmol) and L-[4,5- ^3H]-leucine (sp. radioactivity 60 Ci/mmol) were obtained from du Pont-New England Nuclear (Boston, MA, U.S.A.). [2- ^{14}C]-glycyl[1- ^{14}C]-sarcosine (sp. radioactivity 109 mCi/mmol) was synthesized by Cambridge Research Biochemicals, Ltd. (Billingham, Cleveland, U.K.). 5,5-[2- ^{14}C]-dimethylloxazolidine-2,4-dione (DMO) (sp. radioactivity 55 mCi/mmol) was from American Radiolabeled Chemicals (St. Louis, MO, U.S.A.).

Cell culture

Caco-2 cells were maintained in 75 cm^2 culture flasks at 37 °C in a humidified CO_2/air (1:19) atmosphere. The culture flasks had been coated with collagen (5 $\mu\text{g}/\text{cm}^2$). The culture medium used was MEM with 10 or 20% fetal-bovine serum, 1% penicillin-streptomycin and 1% MEM non-essential amino acids. Cells grown to confluence were released by trypsin treatment (0.25% trypsin and 0.5 mM EDTA in PBS) and subcultured in collagen (4 $\mu\text{g}/\text{cm}^2$)-treated 35 mm disposable Petri dishes (Falcon). On the day before the day of uptake measurement, or when the pretreatments were started, the medium was replaced with a modified culture medium which did not contain penicillin and streptomycin. With a starting cell density of 1×10^6 cells/dish, the Caco-2 cultures reached confluence on day 4 or 5. In most experiments, uptake was measured on the day the cells reached confluence.

Cell treatments

Stock solutions of phorbol esters, staurosporine and cycloheximide were prepared in dimethyl sulfoxide (DMSO). The final concentration of DMSO during treatment was 0.1–0.65%. The control cells were treated with the respective concentration of the solvent in each experiment. The preincubations were performed using the specified concentrations of PMA (0.1–1 μM), PDD (1 μM), PDBu (1 μM), 4 α -phorbol (1 μM), mezerein (0.5 or 1 μM), staurosporine (0.5 μM) or cycloheximide (40 μM) for the desired time (mostly 2 h). After the treatment, the monolayers were washed once with the uptake buffer before initiation of the uptake measurement.

Uptake measurement

Uptake of glycylsarcosine, Na^+ , leucine, alanine or DMO was determined at room temperature as described previously [20–22]. The uptake medium was 25 mM Mes/Tris (pH 6.0), or 25 mM Hepes/Tris (pH 7.5), containing 140 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl_2 , 0.8 mM MgSO_4 and 5 mM glucose unless indicated otherwise. Na^+ -free media were prepared by replacing NaCl in the uptake medium by choline chloride. A modified uptake medium which contained 1 mM NaCl and was iso-osmotically adjusted with choline chloride was used for Na^+ -uptake measurements. The procedure for NH_4Cl pre-pulse was the same as described previously [22]. Uptake was initiated by removing the wash medium from the dish and adding 1 ml of uptake medium containing radiolabelled glycylsarcosine, Na^+ , leucine, alanine or DMO. The final concentration of glycylsarcosine was 10 μM , except for kinetic studies. The non-mediated

component of glycylsarcosine uptake in kinetic experiments was determined by measuring radiolabel uptake from the medium at pH 7.5 in the presence of an excess amount (50 mM) of unlabelled leucylmethionine. After incubation for the desired time (mostly 10 min), the buffer was removed and the monolayers were quickly washed four times with the uptake buffer, dissolved with 1 ml of 0.2 M NaOH containing 1% SDS and transferred to counting vials. The radioactivity associated with the cells was measured by liquid scintillation spectrometry. Leucine incorporation into trichloroacetic acid-insoluble fraction was determined as described previously [21].

Protein determination

For each experiment, the samples for protein measurement were prepared as described earlier [20] and the determination of protein in these samples was done according to the method of Lowry et al. [23].

Statistics

Each experimental point was determined with duplicate or triplicate dishes, and each experiment was repeated at least twice. The results are expressed as means $\pm$ S.E.M. of these replicate values. The kinetic constants were calculated by linear regression of the Eadie–Hofstee plot and confirmed by non-linear regression methods using the Fig. P 6.0 program (Biosoft, Cambridge, U.K.). The calculated parameters are shown with their S.E.M. Statistical analysis was done by the non-parametric two-tailed U-test, and a *P* value of less than 0.05 was considered statistically significant.

RESULTS

Influence of a transmembrane Na^+ gradient or H^+ gradient on glycylsarcosine uptake in Caco-2 cells

The role of a transmembrane Na^+ gradient on the uptake of glycylsarcosine in Caco-2 cells at confluence was studied by measuring the uptake of the dipeptide at a fixed extracellular pH (7.5), but in the presence or absence of Na^+ in the uptake medium. The time course of uptake under these conditions revealed that Na^+ had no influence on the uptake of the dipeptide (Figure 1). The influence of a transmembrane H^+ gradient was studied by measuring the uptake in the presence of NaCl, but at two different extracellular pH values, namely 7.5 and 6.0 (Figure 1). Uptake measured at pH 6.0 was found to be 3–4 times greater than uptake measured at pH 7.5. This stimulation was evident at all time periods studied. These results show that the uptake of glycylsarcosine in Caco-2 cells occurs via an Na^+ -independent process and that the presence of an acidic extracellular pH stimulates this uptake process.

The role of a transmembrane H^+ gradient on the uptake of glycylsarcosine was further analysed by studying the influence of extracellular pH, as well as intracellular pH, on the uptake. The extracellular pH was altered by using uptake media of two different pH values, 7.5 and 6.0. Intracellular pH was altered by NH_4Cl pre-pulse of the cells [22]. The results of these experiments are given in Table 1. With normal intracellular pH, changing the pH of the uptake medium from 7.5 to 6.0 stimulated glycylsarcosine uptake 3.6-fold. This stimulation was almost completely abolished if the intracellular pH was made acidic with an NH_4Cl pre-pulse. These data show that the stimulation observed at an extracellular pH 6.0 was not due to the acidic pH *per se*, but rather due to the presence of an inwardly directed H^+ gradient across the cellular membrane under these conditions. With an

Figure 1 Influence of transmembrane Na⁺ and H⁺ gradients on glycylysarcosine (Gly-Sar) uptake in Caco-2 cells

Uptake of glycylysarcosine (10 μ M) was measured in Caco-2 cells at confluence using three different uptake media: Na⁺-free buffer, pH 7.5 (25 mM Hepes/Tris/140 mM choline chloride/5.4 mM KCl/1.8 mM CaCl₂/0.8 mM MgSO₄/5 mM glucose, ●), Na⁺-buffer, pH 7.5 (25 mM Hepes/Tris/140 mM NaCl/5.4 mM KCl/1.8 mM CaCl₂/0.8 mM MgSO₄/5 mM glucose, ○) and Na⁺-buffer, pH 6.0 (25 mM Mes/Tris/140 mM NaCl/5.4 mM KCl/1.8 mM CaCl₂/0.8 mM MgSO₄/5 mM glucose, ●). The results are given as means $\pm$ S.E.M. for four determinations. When not indicated, the S.E.M. lies within the symbol.

Table 1 Influence of an inwardly directed H⁺ gradient and an outwardly directed H⁺ gradient on glycylysarcosine uptake and Na⁺ uptake in Caco-2 cells

Confluent monolayer cultures of Caco-2 cells were treated for 15 min with either the control buffer (25 mM Hepes/Tris/5.4 mM KCl/1.8 mM CaCl₂/0.8 mM MgSO₄/5 mM glucose/140 mM choline chloride, pH 7.5) or the NH₄Cl-containing buffer (25 mM Hepes/Tris/5.4 mM KCl/1.8 mM CaCl₂/0.8 mM MgSO₄/5 mM glucose/115 mM choline chloride/25 mM NH₄Cl, pH 7.5). After treatment, the monolayers were washed with NH₄Cl-free uptake buffers, and uptake of glycylysarcosine and Na⁺ was measured. The uptake medium used for glycylysarcosine was 140 mM choline chloride/5.4 mM KCl/1.8 mM CaCl₂/0.8 mM MgSO₄/5 mM glucose, buffered with either 25 mM Hepes/Tris, pH 7.5, or 25 mM Mes/Tris, pH 6.0. The concentration of glycylysarcosine was 10 μ M, and uptake was measured with a 15 min incubation. The uptake media used for Na⁺ were the same as those used for glycylysarcosine, except that the media contained, in addition to the regular components, 1 mM NaCl and 0.5 mM ouabain. Uptake of Na⁺ was measured with a 5 min incubation. The uptake measured under conditions of normal intracellular pH and at an extracellular pH of 7.5 was taken as 100%. This value was 0.04 ± 0.01 nmol/15 min per mg of protein for glycylysarcosine and 3.06 ± 0.20 nmol/5 min per mg of protein for Na⁺. The values are means $\pm$ S.E.M. for four determinations.

Intracellular pH	Extracellular pH	Glycylysarcosine uptake (%)	Na ⁺ uptake (%)
Normal	7.5	100 $\pm$ 5	100 $\pm$ 1
	6.0	361 $\pm$ 30	81 $\pm$ 8
Acidic	7.5	56 $\pm$ 2	147 $\pm$ 6
	6.0	116 $\pm$ 4	80 $\pm$ 10

uptake medium pH of 6.0, there exists a transmembrane H⁺ gradient when the intracellular pH is normal. But this gradient is either drastically reduced or completely abolished when the intracellular pH is made acidic. The role of a transmembrane H⁺ gradient on glycylysarcosine uptake in these cells is further supported by the observations that the uptake measured at an extracellular pH of 7.5 was significantly greater at a normal

Figure 2 Influence of growth state of Caco-2 cells on glycylysarcosine (Gly-Sar) uptake

Subcultures of Caco-2 cells were started with 1×10^6 cells/dish, and the uptake of glycylysarcosine (10 μ M) and the protein content were determined on different days up to 9 days after subculturing. Uptake of the dipeptide was measured with a 15 min incubation, and the uptake medium was 25 mM Mes/Tris/140 mM NaCl/5.4 mM KCl/1.8 mM CaCl₂/0.8 mM MgSO₄/5 mM glucose, pH 6.0. Glycylysarcosine uptake is expressed as nmol/15 min per mg of protein (●) and the protein content as mg/dish (○). The values are means $\pm$ S.E.M. for three or four determinations. When not indicated, the S.E.M. lies within the symbol.

intracellular pH than at an acidic intracellular pH. These results clearly demonstrate that the uptake of glycylysarcosine in Caco-2 cells at confluence is stimulated by an inwardly directed H⁺ gradient and inhibited by an outwardly directed H⁺ gradient.

For comparison, uptake of Na⁺ into the cells was measured under identical conditions. Caco-2 cells express Na⁺-H⁺-exchanger activity [24]. The uptake of Na⁺ via the exchanger is expected to be inhibited by an inwardly directed H⁺ gradient and stimulated by an outwardly directed H⁺ gradient. The data given in Table 1 show that the uptake of Na⁺ measured at normal intracellular pH was significantly less at an extracellular pH of 6.0 than at extracellular pH of 7.5. Similarly, the generation of an outwardly directed H⁺ gradient by acidifying the intracellular pH stimulated Na⁺ uptake significantly. These results validate our experimental approach to generate transmembrane H⁺ gradients in the out-to-in direction as well as in the in-to-out direction and support our conclusions on the influence of these gradients on glycylysarcosine uptake.

Influence of growth state of the Caco-2 cultures on glycylysarcosine uptake

After starting subcultures with 1×10^6 cells/dish, uptake of glycylysarcosine and protein concentration were measured in these cultures on different days up to nine days (Figure 2). The protein concentration of the cultures increased as the number of culture days increased. The specific activity of glycylysarcosine uptake, measured in an uptake medium of pH 6.0 and expressed as uptake per mg of protein, also increased with the number of culture days up to 6 days after which there was a significant decrease. The cultures reached confluence on day 5 and thus the specific activity of glycylysarcosine uptake was maximal when the cells were confluent. Continuation of the cultures beyond this stage had no influence on glycylysarcosine uptake. However, since the protein content of the cells continued to increase, there was

Table 2 Substrate specificity of the glycylsarcosine-uptake system in Caco-2 cells

Uptake of radiolabelled glycylsarcosine (10 μ M) was measured in confluent cultures of Caco-2 cells with a 15 min incubation. The uptake medium was 25 mM Mes/Tris/140 mM NaCl/5.4 mM KCl/1.8 mM CaCl_2 /0.8 mM MgSO_4 /5 mM glucose, pH 6.0. The concentration of unlabelled compounds was 10 mM. The uptake of glycylsarcosine measured in the absence of inhibitors was 0.31 ± 0.05 nmol/15 min per mg of protein, and this value was taken as the control (100%). The values given are means $\pm$ S.E.M. for four determinations.

Unlabelled compound	Glycylsarcosine uptake (%)
None	100 ± 3
Glycylsarcosine	16 ± 2
Glycylproline	10 ± 1
Leucylmethionine	8 ± 1
Glycylprolylalanine	17 ± 1
Phenylalanylprolylalanine	14 ± 1
Cephalexin	75 ± 5
Glycine	106 ± 5
Leucine	93 ± 5

Figure 3 Kinetics of the glycylsarcosine-uptake system in Caco-2 cells

Uptake of glycylsarcosine was measured in Caco-2 cells at confluence, with a 10 min incubation over a glycylsarcosine concentration range of 0.25–5 mM. The uptake medium was 25 mM Mes/Tris/140 mM NaCl/5.4 mM KCl/1.8 mM CaCl_2 /0.8 mM MgSO_4 /5 mM glucose, pH 6.0. The diffusional component of the uptake was determined by measuring the uptake in the presence of an excess amount (50 mM) of leucylmethionine, a competing substrate. This component was subtracted from the total uptake to calculate the carrier-mediated uptake which was used in the kinetic analysis. v , uptake rate in nmol/10 min per mg of protein; s , glycylsarcosine concentration in mM. The values are means $\pm$ S.E.M. for four determinations.

a decrease in glycylsarcosine uptake when expressed as uptake per mg of protein.

Substrate specificity of the H^+ -dependent glycylsarcosine uptake system in Caco-2 cells

To determine the substrate specificity of the H^+ -dependent transport system that is responsible for the uptake of glycylsar-

cine in Caco-2 cells, the effect of unlabelled peptides, amino acids and an aminoccephalosporin antibiotic on the uptake of radiolabelled glycylsarcosine was investigated. As shown in Table 2, a variety of dipeptides and tripeptides markedly decreased the uptake of glycylsarcosine, and so did the aminoccephalosporin antibiotic cephalexin. In contrast, the free amino acids glycine and leucine had no effect. These data show that the transport system which catalyses the uptake of glycylsarcosine in these cells recognized dipeptides, tripeptides and aminoccephalosporins as substrates. Free amino acids do not interact with this system.

Kinetics of the H^+ -dependent glycylsarcosine-uptake system in Caco-2 cells

The dependence of the glycylsarcosine uptake rate on the concentration of glycylsarcosine was investigated in Caco-2 cells at confluence to determine the kinetic parameters of the transport system. Uptake rates were measured with a 10 min incubation over a substrate concentration range of 0.25–5 mM. Non-mediated uptake was determined by measuring glycylsarcosine uptake in the presence of an excess amount (50 mM) of leucylmethionine, a competing substrate. Carrier-mediated uptake, which was calculated by subtracting the non-mediated component from the total uptake, was used in the kinetic analysis. The relationship between uptake rate and substrate concentration was found to be hyperbolic (Figure 3, inset), indicating saturability of the transport system. When the results were expressed in the form of an Eadie-Hofstee plot (uptake rate/substrate concentration versus uptake rate), a straight line ($r^2 = 0.99$) was obtained (Figure 3). This suggests the presence of a single transport system catalysing the uptake of glycylsarcosine in these cells. The apparent Michaelis-Menten constant (K_m) was 1.1 ± 0.1 mM and the maximal velocity ($V_{\max}$) was 17.7 ± 0.6 nmol/10 min per mg of protein.

Effects of phorbol esters on glycylsarcosine uptake in Caco-2 cells

Preliminary experiments revealed that treatment of confluent monolayer cultures of Caco-2 cells with PMA, an activator of protein kinase C, resulted in significant inhibition of glycylsarcosine uptake. We performed several initial experiments to determine the conditions necessary to obtain the maximal effect. First, the influence of treatment time on the PMA-induced inhibition was studied at a PMA concentration of 1 μ M. Significant inhibition ($\approx 20\%$) was noticeable with a treatment time as short as 15 min, and maximal inhibition, which varied over the range of 30–50% in different experiments, was reached with a 2 h treatment time. We then performed experiments to study the dose-response relationship by using a treatment time of 2 h. It was found that a PMA concentration of 50 nM was sufficient to cause maximal inhibition. Figure 4 describes the time course of glycylsarcosine uptake in cells treated with or without 100 nM PMA in the culture medium for 2 h prior to uptake measurement. The uptake values in PMA-treated cells were 30–35% less than those in control cells which had been treated only with DMSO. The inhibition was noticeable at all time periods. Under both conditions, uptake was linear at least up to 10 min. An incubation time of 10 min was used in subsequent experiments to determine initial uptake rate.

Table 3 provides evidence for the specificity of protein kinase C activation to produce the inhibitory effect. PMA and PDBu, both being active phorbol esters which are able to cause activation of protein kinase C, inhibited glycylsarcosine uptake. Mezerein, a phorbol ester analogue which has the ability to activate protein

Figure 4 Effect of PMA on the time-dependent uptake of glycylsarcosine (Gly-Sar) in Caco-2 cells

Confluent monolayer cultures of Caco-2 cells were treated for 2 h with (○) or without (●) 100 nM PMA in culture medium. Following the treatment, uptake of glycylsarcosine (10 μ M) was measured at pH 6.0 in a NaCl-containing buffer. The values are means $\pm$ S.E.M. for four determinations. When not shown, the S.E.M. lies within the symbol.

Table 3 Influence of protein kinase C activators on glycylsarcosine uptake in Caco-2 cells

Uptake of radiolabelled glycylsarcosine (10 μ M, 10 min, pH 6.0) was measured in confluent cultures of Caco-2 cells after treatment with DMSO (control) or the following compounds (1 μ M) for 2 h in culture medium: PMA, PDBu, mezerein, PDD and 4 α -phorbol. The values given are means $\pm$ S.E.M. for four determinations. Abbreviation: NS, not significant (*P* values compared with control uptake).

Compound	Glycylsarcosine uptake	
	(pmol/10 min per mg of protein)	(%)
Control	149.0 $\pm$ 9.3	100
PMA	77.1 $\pm$ 4.2	52 (<i>P</i> < 0.05)
PDBu	96.8 $\pm$ 8.6	65 (<i>P</i> < 0.05)
Mezerein	97.3 $\pm$ 5.5	65 (<i>P</i> < 0.05)
PDD	153.5 $\pm$ 8.1	103 (NS)
4 α -phorbol	152.3 $\pm$ 7.2	102 (NS)

kinase C, also caused significant inhibition. In contrast, PDD, which is an inactive phorbol ester and has no effect on protein kinase C [25], failed to have any effect. Similarly, 4 α -phorbol also was unable to inhibit glycylsarcosine uptake.

Influence of staurosporine on the PMA- and mezerein-induced inhibition of glycylsarcosine uptake

To determine the role of protein kinase C in the inhibition of glycylsarcosine uptake caused by active phorbol esters, we studied the influence of staurosporine on the PMA- and mezerein-induced inhibition. Staurosporine is an inhibitor of protein kinase C. If the inhibition of glycylsarcosine uptake caused by phorbol esters were due to activation of protein kinase C, staurosporine should be able to attenuate or completely block this inhibition. The data given in Table 4 clearly show that the

Table 4 Effect of staurosporine on the PMA- or mezerein-induced inhibition of glycylsarcosine uptake in Caco-2 cells

Uptake of radiolabelled glycylsarcosine (10 μ M, 10 min, pH 6.0) was measured in confluent cultures of Caco-2 cells after treatment with the listed compounds (0.5 μ M) for 2 h in culture medium. The values given are means $\pm$ S.E.M. for four to eight determinations. Abbreviation: NS, not significant (*P* values compared with control uptake).

Compound	Glycylsarcosine uptake	
	(pmol/10 min per mg of protein)	(%)
Control	134.1 $\pm$ 7.1	100
PMA	89.0 $\pm$ 3.0	66 (<i>P</i> < 0.05)
Staurosporine	141.8 $\pm$ 2.6	106 (NS)
PMA + staurosporine	139.2 $\pm$ 3.8	104 (NS)
Mezerein	95.8 $\pm$ 3.0	71 (<i>P</i> < 0.05)
Mezerein + staurosporine	136.3 $\pm$ 2.7	102 (NS)

Table 5 Effects of PMA on the transport of leucine and alanine in Caco-2 cells

After 2 h treatment with PMA (100 nM), uptake of radiolabelled substrates was measured in confluent monolayer cultures of Caco-2 cells using a 10 min incubation. Concentrations were: glycylsarcosine, 10 μ M; L-leucine, 10 nM; L-alanine, 20 nM. Uptake of glycylsarcosine was measured from a NaCl-containing uptake buffer at pH 6.0, that of leucine from a Na⁺-free uptake buffer at pH 7.5 and that of L-alanine from a NaCl-containing uptake buffer at pH 7.5. Abbreviation: NS, not significant (*P* values compared with respective controls). Values given represent means $\pm$ S.E.M. for three to five determinations.

Substrate	Uptake		
	(pmol/10 min per mg of protein)		
	Control	PMA	(%)
Glycylsarcosine	141.20 $\pm$ 3.2	97.90 $\pm$ 2.9	69 (<i>P</i> < 0.05)
L-Leucine	1.07 $\pm$ 0.04	1.01 $\pm$ 0.02	94 (NS)
L-Alanine	0.69 $\pm$ 0.01	0.54 $\pm$ 0.01	78 (<i>P</i> < 0.05)

inhibition induced by PMA and mezerein was effectively blocked by co-treatment of the cells with staurosporine. These results strongly support the suggestion that activation of protein kinase C in Caco-2 cells results in the inhibition of the peptide/H⁺ co-transport system.

Specificity of the PMA-induced inhibition of glycylsarcosine uptake

Further experiments were performed to determine whether the phorbol ester-induced inhibition of glycylsarcosine uptake could be due to a generalized non-specific effect. In these experiments, uptake of two amino acids, leucine and alanine, was measured in parallel with glycylsarcosine uptake in control and in PMA-treated cells (Table 5). It was found that PMA, under the same treatment conditions in which it caused significant inhibition of glycylsarcosine uptake, failed to have any effect on leucine uptake. However, uptake of alanine was inhibited to an appreciable extent. Since leucine uptake was not inhibited by PMA, the data suggest that the inhibition of glycylsarcosine uptake by PMA could not be a non-specific effect.

Figure 5 Influence of PMA on the kinetic parameters of glycylsarcosine uptake in Caco-2 cells

Confluent monolayer cultures of Caco-2 cells were treated with (○) or without (●) 100 nM PMA for 2 h in culture medium. Following the treatment, the kinetic parameters of the glycylsarcosine uptake system were analysed in control and in PMA-treated cells, as described in the legend to Figure 3. The results are given as Eadie-Hofstee plots (v versus v/s). v , uptake rate in nmol/10 min per mg of protein; s , glycylsarcosine concentration in mM. Values are means $\pm$ S.E.M. for four determinations.

Influence of PMA on the kinetic parameters of glycylsarcosine uptake

In order to determine the effects of PMA on the kinetic parameters of glycylsarcosine uptake, cells were treated for 2 h with or without PMA (100 nM) in the cell culture medium prior to the kinetic studies. The kinetic constants for glycylsarcosine uptake were calculated in control and in PMA-treated cells. The uptake rate, after correction for the contribution by non-mediated processes, was hyperbolically related to the concentration of glycylsarcosine, and the relationship obeyed Michaelis-Menten kinetics describing a single mediated process in control cells as well as in PMA-treated cells. The experimental data were transformed into a linear format by the Eadie-Hofstee method and are shown in Figure 5. Linear plots (uptake rate/glycylsarcosine concentration versus uptake rate) with regression coefficients of -0.987 (control) and -0.984 (PMA) respectively were obtained, thus supporting the involvement of a single transport system. The data show that PMA elicited alterations in the maximal velocity of glycylsarcosine transport without changing the affinity of the carrier for glycylsarcosine. In control cells, the apparent Michaelis-Menten constant (K_t) for the uptake process was 1.2 ± 0.1 mM and the maximal velocity ($V_{\max}$) was 16.5 ± 0.6 nmol/10 min per mg of protein. In the PMA-treated cells, the K_t was 1.1 ± 0.1 mM and the $V_{\max}$ was 10.6 ± 0.4 nmol/10 min per mg of protein.

Possible mechanism of the PMA-induced inhibition of the peptide-transport system

Theoretically, there are several mechanisms by which activation of protein kinase C could lead to inhibition of the peptide/ H^+ co-transport system. One possible mechanism is that treatment of the cells with PMA inhibits *de novo* synthesis of the transport protein and thereby reduces its density in the plasma membrane. To evaluate this possibility, we studied the effect of PMA in the absence or presence of cycloheximide, an inhibitor of cellular protein synthesis. The monolayers were treated for 2 h with

Table 6 Influence of cycloheximide on the PMA-induced inhibition of glycylsarcosine transport and on the incorporation of leucine into trichloroacetic acid-insoluble fraction in Caco-2 cells

Confluent monolayer cultures of Caco-2 cells were treated with DMSO (control) or cycloheximide (40 μ M) in the absence or presence of PMA (100 nM) for 2 h in culture medium. Following the treatment, uptake of glycylsarcosine (10 μ M) was measured at pH 6.0 using a 10 min incubation, and incorporation of [^{3} H]leucine (1 μ Ci/ml) into trichloroacetic acid-insoluble fraction was measured at pH 7.5 using a 2 h incubation. Values given represent means $\pm$ S.E.M. for three or four determinations. Values in parentheses are percentages of corresponding control values.

	Glycylsarcosine uptake (pmol/10 min per mg of protein)		Leucine incorporation (c.p.m./2 h per mg of protein)
	— PMA	+ PMA	
Control	129.6 $\pm$ 1.4 (100)	76.6 $\pm$ 1.1 (59)	34 834 $\pm$ 1771 (100)
Cycloheximide	126.7 $\pm$ 5.6 (100)	77.0 $\pm$ 1.0 (61)	4380 $\pm$ 23 (13)

cycloheximide (40 μ M) in the presence or absence of PMA (100 nM) in culture medium, and the uptake of glycylsarcosine was measured at pH 6.0. The results given in Table 6 demonstrate that cycloheximide did not affect glycylsarcosine transport in the absence of PMA. More importantly, the PMA-induced inhibition of glycylsarcosine transport remained unaffected following co-treatment of the cells with cycloheximide. To demonstrate that cycloheximide did block cellular protein synthesis effectively under these experimental conditions, we measured incorporation of leucine into trichloroacetic acid-insoluble fraction. As shown in Table 6, cycloheximide decreased the leucine incorporation by 87%.

PMA is known to influence pH-regulatory systems such as the Na^+ - H^+ exchanger in a variety of cells. Therefore it is possible that PMA alters the transmembrane pH gradient by acidifying the intracellular pH via this mechanism, and such an effect could be expected to reduce the driving force for the peptide/ H^+ co-transport, thus leading to a decrease in glycylsarcosine transport. To investigate whether acidification of intracellular pH by possible inhibition of the Na^+ - H^+ exchanger by PMA might have contributed to the observed inhibition of glycylsarcosine transport, we studied the effect of PMA on glycylsarcosine transport by treating the cells with PMA in a Na^+ -free medium in which NaCl was iso-osmotically replaced with choline chloride. It was found that the PMA-induced inhibition of glycylsarcosine transport remained intact even under these Na^+ -free conditions, indicating that the inhibition could not have been due to any possible influence of PMA on the Na^+ - H^+ exchanger. This conclusion is further supported by a recent study by Watson et al. [26], which showed that the Na^+ - H^+ exchanger in Caco-2 cells is not affected by phorbol esters.

In addition to the Na^+ - H^+ exchanger, many other transport systems also participate in the regulation of intracellular pH. To demonstrate unequivocally that the PMA-induced inhibition of glycylsarcosine transport in Caco-2 cells is a direct effect on the transporter rather than a consequence of PMA-mediated alterations in the transmembrane H^+ gradient which is the driving force for the transport system, we measured the intracellular pH in control and in PMA-treated cells from the equilibrium uptake of DMO, a weak organic acid. This technique has been widely used to determine intracellular pH in a variety of systems [27]. In this experiment, the cells were treated with or without 100 nM PMA at 37 $^{\circ}$ C for 2 h in an Na^+ -free medium, pH 7.5. Following the treatment, the uptake of DMO (0.5 μ Ci/ml) was measured

using the same medium. Caco-2 cells, when grown on an impermeable plastic support in the presence of serum and Na⁺, form domes as a result of transepithelial transport of fluid [13]. Since DMO is expected to equilibrate not only in the intracellular medium but also in the fluid contained within the domes, uptake of DMO under these conditions would lead to erroneous values for intracellular pH. Therefore, we used a medium containing choline chloride instead of NaCl during treatment with PMA. Examination of the cultures under a microscope revealed the absence of domes under these conditions. Since the PMA-induced inhibition of the transport of glycylsarcosine remains unaltered whether the treatment with PMA is done in an NaCl buffer or in a choline chloride buffer, this experimental approach is valid. The time course of DMO uptake under these conditions showed that the uptake reached equilibrium within 2.5 min of incubation and remained unaltered over a 20 min period. DMO uptake measured with a 10 min incubation was therefore employed to calculate the intracellular pH, using a value of 3.65 $\mu\text{l}/\text{mg}$ of protein for the intracellular volume [28,29], and a value of 6.27 for the pK for DMO under the experimental conditions [30]. The calculated value for the intracellular pH in these experiments was 7.31 ± 0.02 ($n = 8$) for control cells and 7.34 ± 0.01 ($n = 8$) for PMA-treated cells. The difference between these two values was not statistically significant ($P = 0.328$). These values agree well with those reported for Caco-2 cells in other laboratories using different experimental techniques [24,26,31]. These data demonstrate convincingly that the PMA-induced inhibition of glycylsarcosine transport in Caco-2 cells was not due to alterations in the transmembrane H⁺ gradient.

DISCUSSION

The human colon-carcinoma cell line Caco-2 has been used by other investigators to characterize the transport of aminocephalosporin antibiotics [10–12], a process which has been convincingly shown in several studies to occur via the H⁺-dependent peptide-transport system [6–8]. Transport of these antibiotics in this cell line is stimulated by the presence of an inwardly directed H⁺ gradient, is inhibited by several peptides, but not by free amino acids, and is sensitive to metabolic poisons. However, there has been no direct study to demonstrate transport of peptides in these cells. Since the transport of cephalosporin antibiotics can occur via transport systems other than the peptide transport system [32,33], direct demonstration of the ability of these cells to accumulate peptides in response to a transmembrane H⁺ gradient would provide unequivocal evidence for the expression of the peptide-H⁺ co-transporter. The present study was designed to investigate the characteristics of the transport of the dipeptide glycylsarcosine in these cells. The results of the study show that transport of glycylsarcosine in Caco-2 cells is Na⁺-independent, but is markedly stimulated by an inwardly directed H⁺ gradient. The H⁺-dependent transport of glycylsarcosine is inhibited by dipeptides and tripeptides but not by free amino acids. Cephalixin, a cephalosporin antibiotic, is also able to compete with this transport system. The Michaelis–Menten constant for the transport of glycylsarcosine via this system is 1.1 mM. The study also provides unequivocal evidence for the involvement of a transmembrane H⁺ gradient in the transport of peptides. Previous studies on the transport of cephalosporin antibiotics in Caco-2 cells have demonstrated that the transport is sensitive to extracellular pH, the transport being maximal around pH 6.0 [10–12]. This was taken as supportive evidence for the H⁺-dependence of the transport system. In the present investigation, the role of a transmembrane H⁺ gradient in the transport of glycylsarcosine

was assessed by altering not only the extracellular pH, but also the intracellular pH. In addition, the influence of these pH manipulations on the transport of the peptide was compared with the influence of similar manoeuvres on the transport of Na⁺. The transport of a peptide via the peptide/H⁺ co-transporter is expected to be stimulated by an inwardly directed H⁺ gradient and inhibited by an outwardly directed H⁺ gradient. On the other hand, the transport of Na⁺ via the Na⁺–H⁺ exchanger is expected to be inhibited by an inwardly directed H⁺ gradient and stimulated by an outwardly directed H⁺ gradient. Thus the studies reported in the present investigation on the effect of transmembrane H⁺ gradients on the uptake of Na⁺ and of glycylsarcosine allow us to determine unambiguously the role of a H⁺ gradient in peptide transport. The results clearly show that the transport of glycylsarcosine in Caco-2 cells is stimulated by an inwardly directed H⁺ gradient. Determination of the intracellular concentration of glycylsarcosine at the end of a 30 min incubation in the presence of an inwardly directed H⁺ gradient revealed that the peptide is accumulated in these cells against a concentration gradient. The intracellular volume was assumed to be approx. 3.65 $\mu\text{l}/\text{mg}$ of protein in these calculations [28,29]. With a 30 min incubation, the intracellular concentration of glycylsarcosine was $142 \pm 3 \mu\text{M}$, while the concentration of the dipeptide in the uptake medium was 10 μM . This is clear evidence for energization of uphill peptide transport in these cells by an inwardly directed H⁺ gradient.

An additional purpose of the present study was to use the Caco-2 cells as a model to investigate regulation of intestinal peptide transport. The results presented here provide evidence for the first time for regulation of the peptide/H⁺ co-transport system by protein kinase C. Treatment of cells with activators of protein kinase C (PMA, PDBu and mezerein) resulted in significant inhibition of the transport system, an effect not seen with PDD and 4 α -phorbol, which do not activate protein kinase C. The inhibition was not due to some non-specific effect, because transport of leucine was not affected in these cells under identical conditions. The involvement of protein kinase C in this inhibition is strongly indicated, because the inhibitory effect could be completely blocked by staurosporine, an inhibitor of protein kinase C. Kinetic analysis shows that the protein kinase C-induced inhibition is associated with a decrease in the maximal velocity of the transport system with no change in the affinity of the system for its substrates.

Theoretically there are several mechanisms by which activation of protein kinase C could lead to inhibition of the peptide/H⁺ co-transport system. Activation of protein kinase C may block the *de novo* synthesis of the transporter protein, thereby reducing the density of the transporter molecules in the plasma membrane. This, however, does not seem to be the case, because the PMA-induced inhibition of the peptide transporter activity was not prevented by cycloheximide, an inhibitor of cellular protein synthesis. The inhibitory effect was also not due to alterations in the transmembrane pH gradient, the driving force for the transporter, because there was no evidence for any change in the intracellular pH as a result of PMA treatment. A more likely possibility is that the peptide transporter is regulated by post-translational modification involving phosphorylation/dephosphorylation. A change in the phosphorylation state of the transport protein either directly by the action of protein kinase C itself or indirectly with the involvement of additional protein kinases/phosphatases could result in the inhibition of its catalytic function.

We have recently described how the function of the taurine transporter expressed in HT-29 cells is also regulated by protein kinase C [21]. Activation of protein kinase C by PMA treatment

in these cells leads to inhibition of the taurine transporter. In this case also the inhibition seems to be the result of a direct effect of protein kinase C on the transporter. It appears that several nutrient transport systems in the small intestine may be under the regulation of protein kinase C.

In conclusion, the present investigation provides unequivocal evidence for the expression of the peptide/H⁺ co-transport system in the human colon-carcinoma cell line Caco-2. After completion of the work presented here and during preparation of this article, Thwaites et al. [31] reported that transport of glycylsarcosine in Caco-2 cells is accompanied by cytosolic acidification, indicating co-transport of the dipeptide with H⁺. This supports the conclusion of the present study with regard to the role of a transmembrane H⁺ gradient as the driving force for peptide transport in Caco-2 cells. The demonstration in the present study that the function of the transport system in these cells is inhibited by agents which activate protein kinase C strongly suggests that the peptide transport system in the normal intestine may be under the regulatory control of protein kinase C.

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