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A. Scholl
M. Gent
H. Daniel

Alanine transport across the human placetal brush border membrane and the role of SH groups in carrier function

Charakteristik des Alanintransports an der Bürstensaummembran der menschlichen Plazenta und Identifizierung von freien SH-Gruppen in den Transportproteinen

Summary We have determined the kinetic characteristics of alanine transport into brush border membrane vesicles (BBMV) of human full term placenta and identified functional groups of the carrier proteins that are important for transport function. Alanine influx into BBMV was found to be mediated by two transport systems with different kinetic features and distinct substrate specificities. An uphill operating electrogenic Na^+-dependent cotransport system could be kinetically separated from a Na^+-independent facilitated diffusion system. The Na^+-dependent transporter mediates Na^+-alanine cotransport with a 1:1 flux coupling ratio (Hill coefficient 1.13 ± 0.12) and a Km for alanine of 0.45 ± 0.06 mmol/l. Halfmaximal stimulation of Na^+-dependent alanine influx was observed at a Na^+ concentration (NaCl) of 51.4 ± 1.3 mmol/l. A variety of group specific reagents were used to identify functional groups in the transport proteins. Only compounds reacting with SH-residues (NEM, DTNB, PCMBS) or NH_2-groups (PITC) were found to affect Na^+ dependent and Na^+ independent alanine transport. The EC_{50} value for inhibition of alanine influx by PCMBS was 450 ± 48 µmol/l. Chemical modifications of SH-groups by PCMBS caused a significant reduction ($p < 0.005$) in the V_{max} for Na^+-dependent alanine influx from 0.57 ± 0.06 to 0.16 ± 0.05 nmol·mg protein^{-1}·10s^{-1} without affecting significantly the Km value. Inhibition by PCMBS was reversed by treatment of BBMV with DTT. When the substrate binding site of the transporter was protected by alanine or leucine, PCMBS still blocked transport function, indicating that the crucial SH groups are not located within the substrate binding site of the transport proteins.

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A. Scholl · Prof. Dr. H. Daniel (✉)
AG Biochemie der Ernährung
Institut für Ernährungswissenschaft
Justus-Liebig-Universität Gießen
Wilhelmstraße 20
35392 Gießen

PD Dr. M. Gent
Kreiskrankenhaus Lich
Goethestraße 4
35423 Lich

Zusammenfassung Transportsysteme in der apikalen Zellmembran der Syncytiothrophoblasten der Plazenta sind für den Nährstofftransport aus dem mütterlichen Kreislauf in den fetalen Stoffwechsel von elementarer Bedeutung für Wachstum und Entwicklung des Fötus. Die Carriersysteme für Aminosäuren limitieren dabei die Versorgung des Fötus mit essentiellen und nicht essentiellen Aminosäuren. An isolierten Bürstensaummembranvesikeln (BBMV) reifer menschlicher Placenta haben wir die Aufnahme von neutralen Aminosäuren am Beispiel von Alanin charakterisiert. Darüber hinaus wurden durch den Einsatz gruppenspezifischer Reagentien wichtige Aminosäureseitengruppen der entsprechenden Transportproteine identifiziert. Die Alaninaufnahme in die BBMV wird durch zwei sättigbare Carriersysteme mit unterschiedlichen Eigenschaften vermittelt. Neben einem elektrogenen Na^+-abhängigen System, das auch Gln, meAIB und Met transportiert, existiert ein Na^+-unabhängiges System, das kein meAIB, dafür aber zusätzlich Leu und Tyr transportiert. Beide Transportsysteme haben gleiche Affinitätskonstanten (Km-Werte) für Alanin (0,45 ± 0,06 mmol/l). Das Na^+-abhängige System transportiert Alanin mit einer 1:1 Stöchiometrie mit Na^+-Ionen und weist eine 3fach höhere maximale Transportgeschwindigkeit auf.

Durch Einsatz gruppenspezifischer Reagentien wurden freie SH-Gruppen in den beiden Carriern identifiziert, die für die Funktion von entscheidender Bedeutung sind. Durch Oxidation und Rückreduktion der SH-Gruppen konnte die Transportleistung der Carrier reversibel verändert werden. Da die modifizierenden Reagentien die SH-Gruppen auch in Gegenwart der Substrate blockierten, ist zu vermuten, daß die wichtigen SH-Gruppen nicht in der Substratbindungsstelle der Transportproteine liegen.

Key words Human placenta – brush border membrane – alanine transport – group specific reagents – SH-groups

Schlüsselwörter Alanintransport – menschliche Plazenta – Kinetik – gruppenspezifische Reagentien – SH-Gruppen

Introduction

The placenta is the site of absorption for nutrients required by the fetus for growth and development. Nutrients enter the fetus by uptake from the maternal blood across the brush border membrane of the syncytiotrophoblasts. A variety of specialized transport systems is responsible for uptake of sugars, amino acids, dipeptides, vitamines and electrolytes (2, 15, 16). With respect to amino acid uptake into human syncytiotrophoblasts studies on brush border membrane vesicles have identified a series of Na^+-dependent and Na^+-independent transport systems with overlapping specificity (1, 5, 13, 17, 18). Although some of these transport systems have been studied with respect to kinetic features and substrate specificity (1, 7, 8, 9, 10, 11), physicochemical characteristics of the proteins mediating transmembrane transport of amino acids have not been determined. As shown for other nutrient and electrolyte transport systems, group specific agents can successfully be employed to identify functional groups in the transport proteins which are essential for transport function (3, 4, 6, 12). Here we describe the effects of a variety of group specific reagents on uptake of alanine via a Na^+-dependent and a Na^+-independent transport system in human placental brush border membrane vesicles.

Materials

L-[3-^{3}H]-Alanine (2.84 BGq/mmol; 37 MBq/ml) and L-[3-^{3}H]-Glutamate (0.71 GBq/mmol; 37 MBq/ml) were purchased from Du Pont-De Nemours, Dreieich, FRG. p-chloromercuribenzenesulfonate (PCMBS), dicyclohexylcarbodiimide (DCCD), diethylprocarbonate (DEP), 5,5'-dithiobis(2-nitro-benzoic acid) (DTNB), N-ethylmaleimide (NEM), N-acetylimidazole (NAI), phenylglyoxal (PGO), phenylisothiocyanate (PITC), phenylmethylsulfonylfluoride (PMSF) and dithiotreitol (DTT) were supplied by Sigma, Deisenhofen, FRG. All other chemicals were of the highest purity available.

Methods

Preparation of membrane vesicles

Brush border membrane vesicles (BBMV) from human placenta were prepared according to lioka et al. (9). Full term placenta was obtained from the Kreiskrankenhaus Lich, washed immediately after delivery with 0.9 % NaCl and kept on ice for transport to the laboratory. Preparation of membrane vesicles was generally carried out within 1 hour after delivery. The marker enzymes for the brush border membrane, alkaline phosphatase (EC 3.1.3.1.) and 5'-nucleotidase (EC 3.1.3.5.) were enriched 20 ± 3 and 14 ± 3 times, respectively, when compared to the homogenate of the washed placental tissue. There was no significant enrichment of Na^+/K^+-ATPase. BBMV were loaded, if not stated otherwise, with a buffer containing 20 mmol/l HEPES/Tris (pH 7.5), 280 mmol/l mannitol and 0.25 mmol/l $CaCl_2$. In uptake studies with glutamate all loading buffers contained 100 mmol/l KCl replacing 200 mmol/l mannitol. The protein concentration of the vesicle suspension was adjusted to 6 mg/ml and BBMV were stored in liquid nitrogen until use.

Treatment of BBMV with group specific reagents

For treatment of membrane vesicles with the various modifying agents, placental BBMV were incubated at pH 7.5 (20 mmol/l HEPES/Tris, 280 mmol/l mannitol, 0.25 mmol/l $CaCl_2$) for 1 hour at 25 °C with the SH-specific compounds DTNB, PCMBS, NEM, the disulfide modifying DTT; the arginine modifying PGO and the tyrosine modifying NAI. Treatment with the histidyl-group specific reagent DEP was performed at pH 6.4 (20 mmol/l potassium phosphate buffer, 280 mmol/l mannitol, 0.25 mmol/l $CaCl_2$) and treatment with the NH_2-selective PITC and the carboxy-group-specific DCCD was done at pH 7.5 in phosphate buffers. Stock solutions of the compounds were made in ethanol followed by dilution with the buffers. The concentration of ethanol during pretreatment of the BBMV was always kept below 3 % (v/v), a concentration found not to affect transport function (data not shown). Equal concentrations of ethanol were added to the corresponding controls. After treatment, the reaction mixture was diluted with the preloading buffer (20 mmol/l HEPES/Tris buffer, 280 mmol/l mannitol, 0.25 mmol/l $CaCl_2$, pH 7.5). BBMV were collected by centrifugation and washed again in the same buffer. The final membrane pellets were suspended in a small volume of preloading buffer and protein concentration was adjusted to 6 mg/ml. Substrate protection experiments were carried out with selected group specific reagents in the

presence of 2 mmol/l alanine or leucine added to the reaction mixture prior to the addition of the modifying agents. In these experiments, control membrane vesicles were treated with the protective substrates in the absence of group specific reagents.

Transport studies

Uptake measurements with ^{3}H-alanine and ^{3}H-glutamate were performed at 25 °C by using a Millipore filtration technique with membrane filters of 0.45 µm pore size (BA 85, Schleicher & Schüll, Düren, FRG). Uptake was initiated by mixing 20 µl of vesicle suspension with 80 µl of incubation medium containing 20 mmol/l HEPES/Tris buffer, pH 7.5, 80 mmol/l mannitol, 0.25 mmol/l $CaCl_2$, 100 mmol/l NaCl or 100 mmol/l KCl. Uptake was terminated by a 50-fold dilution with an ice cold stop solution (5 mmol/l HEPES/Tris buffer, 155 mmol/l KCl, pH 7.5) followed by filtration. For kinetic analysis a 10 s incubation was employed to measure initial rate uptake since alanine influx was found to be linear at least up to 0.5 min.

Calculations and statistics

All calculations (linear as well as non-linear regression analysis) were performed by using INPLOT (GraphPAD, Los Angeles, CA). The experiments were generally carried out in triplicate with at least 2 membrane preparations and results are presented as the mean ± SEM. Significance of differences between the uptake rates and constants were determined by a non-paired t-test.

Results

Transport of alanine into osmotically active BBMV as a function of time

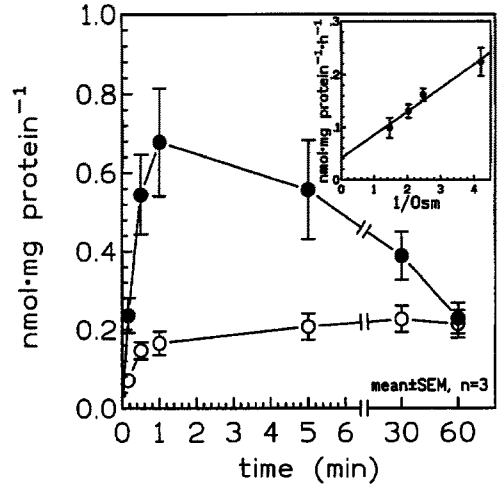
When uptake of 0.15 mmol/l of ^{3}H-alanine into BBMV was measured as a function of time in the presence of an inwardly directed Na^+ gradient ($NaCl_{in}$ 0/$NaCl_{out}$ 100 mmol/l) a pronounced overshoot phenomenon was observed. In contrast, when BBMV were incubated in the presence a K^+ gradient (KCl_{in} 0/KCl_{out} 100 mmol/l), no overshoot phenomenon was obtained and uptake reached equilibrium values at 30 min of incubation (Fig. 1). That the Na^+ gradient dependent influx of alanine occured into an osmotically reactive space was demonstrated by osmotic shrinkage of BBMV in the presence of increasing concentrations of mannitol in the extravesicular medium (inset to Fig. 1). Initial uptake of alanine in the presence of a NaCl gradient was further elevated when an outwardly directed K^+-diffusion potential was imposed by use of valinomycin in BBMV preloaded with 50 mmol/l K_2SO_4 (data not shown). This indicates that Na+ dependent alanine influx is of rheogenic nature.

Kinetics of alanine transport into BBMV as a function of substrate concentration and its substrate specificity

Initial rate uptake of ^{3}H-alanine in the presence of transmembrane NaCl or KCl gradients were determined as a function of increasing concentrations of alanine (0.1 to 5 mmol/l). Uptake data (Fig. 2) were subjected to non-linear regression analysis by the least square method based on a Michaelis-Menten equation including a linear term. The slope of the linear term was found to be identical (0.06 ± 0.02 versus 0.07 ± 0.02 nmol mg protein^{-1}·10s^{-1}·[S]) under both incubation conditions. A transformation of uptake data according to Eadie-Hofstee after substraction of the linear term is shown in Fig. 2b. A saturable transport kinetics is obtained in the presence of a Na^+ gradient as well as in the presence of a K^+ gradient. The Na^+ dependent system has a K_m of 0.45 ± 0.08 mmol/l and a V_{max} of 0.46 ± 0.09 nmol mg protein^{-1} ·10s^{-1}. The kinetic constants of the transporter operating in the presence of a K^+ gradient were found to be K_m: 0.425 ± 0.064 mmol/l and V_{max}: 0.21 ± 0.08 nmol · mg protein^{-1}·10s^{-1}. Although both systems have identical affinity constants, the V_{max} of the Na^+ dependent transporter is about twofold higher. This indicates that Na^+-ions do not alter substrate affinity but serve specifically

Fig. 1 Uptake of alanine into placental BBMV as a function of incubation time and medium osmolarity

Brush border membrane vesicles (BBMV) from human placenta were loaded with a buffer containing 20 mmol/l HEPES/Tris, 280 mmol/l mannitol and 0.25 mmol/l $CaCl_2$, pH 7.5. Uptake studies were initiated by mixing 20 µl BBMV with 80 µl incubation medium containing 20 mmol/l HEPES/Tris buffer pH 7.5, 80 mmol/l mannitol, 0.25 mmol/l $CaCl_2$, 0.15 mmol/l alanine and 100 mmol/l NaCl (●) or 100 mmol/l KCl (○). **Inset:** Na^+-dependent uptake of alanine at 30 sec of incubation as a function of medium osmolarity. Uptake was performed as described above in the presence of 100 mmol/l NaCl and increasing concentrations of mannitol. All data represent the mean ± SEM of 3 preparations in triplicate.



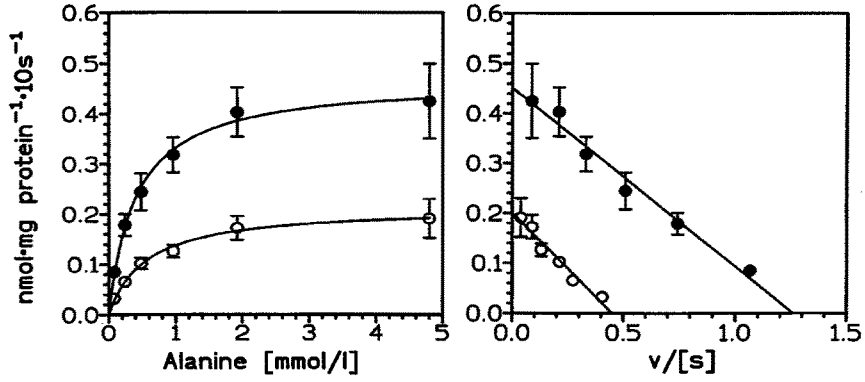


Fig. 2 Initial rate uptake of alanine into BBMV as a function of substrate concentration in the presence of an inwardly directed NaCl or KCl gradient

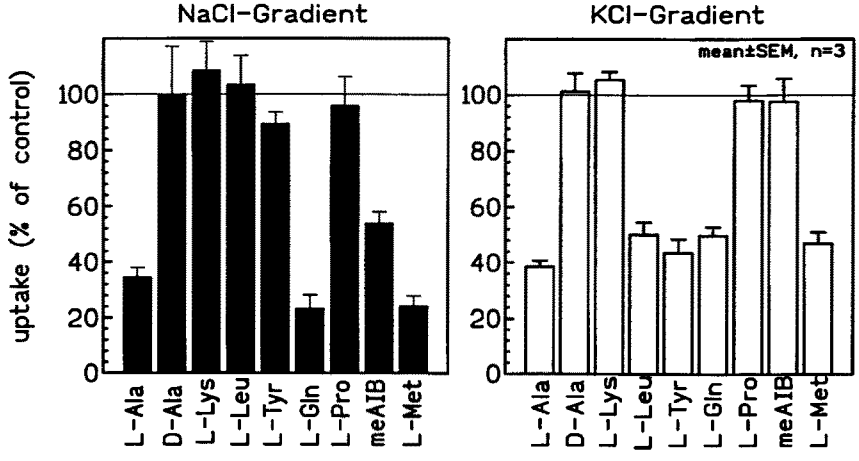
BBMV were preloaded with a buffer containing 20 mmol/l HEPES/Tris 280 mmol/l mannitol and 0.25 mmol/l $CaCl_2$, pH 7.5. Uptake was measured for 10 s of incubation in a medium consisting of 20 mmol/l HEPES/Tris buffer pH 7.5, 80 mmol/l mannitol, 0.25 mmol/l $CaCl_2$, 100 mmol/l NaCl (●) or 100 mmol/l KCl (○) and 0.1 mmol/l alanine to 5 mmol/l alanine. Uptake rates were submitted to non-linear regression analysis based on a Michaelis-Menten equation including a linear term and carrier mediated uptake is shown for the Na^+ dependent and Na^+ independent uptake. Linear regression analysis from the uptake curves plotted according to Eadie-Hofstee (right graph) was applied to determine the corresponding kinetic constants. Data shown represent mean ± SEM of 3 preparations in triplicate.

as the cotransported ion species. The lack of an overshoot in alanine uptake in the presence of a K^+ gradient indicates that the Na^+ independent transporter operates under this experimental conditions downhill most likely as a facilitated diffusion system.

The substrate specificities of the two saturable transport systems were investigated by inhibition experiments with 0.1 mmol/l ^{3}H-alanine as a substrate and selected amino acids (1 mmol/l) serving as inhibitors (Fig. 3). Stereospecific alanine influx by either system was established by the lack of inhibition of L-alanine uptake in the presence of D-alanine. In the presence of a Na+ gradient only glutamine, methionine and the synthetic substrate methyl aminobutyric acid (meAIB) significantly ($p < 0.001$) inhibited alanine influx. The Na^+ independent system was in addition inhibited by leucine and tyrosine but not by meAIB. Proline and lysine did not interact with either system. Based on these experiments leucine appears to be a useful substrate to discriminate the Na^+ independent transporter when measuring uptake of alanine in the presence of an inwardly directed Na^+ gradient.

Fig. 3 Interaction of selected amino acids with Na^+ dependent and Na^+ independent alanine uptake into placental BBMV

Placental BBMV loaded with a buffer containing 20 mmol/l HEPES/Tris buffer, 280 mmol/l mannitol and 0.25 mmol/l $CaCl_2$, pH 7.5 were incubated for 10 s in medium consisting of 20 mmol/l HEPES/Tris buffer pH 7.5, 80 mmol/l mannitol, 100 mmol/l NaCl (filled bars) or 100 mmol/l KCl (open bars), 0.25 mmol/l $CaCl_2$, 0.1 mmol/l alanine and 1 mmol/l of the competing free amino acids. Residual uptake in the presence of inhibitors is expressed as percent of control uptake by either system and is represented as the mean ± SEM of 3 preparations in triplicate.



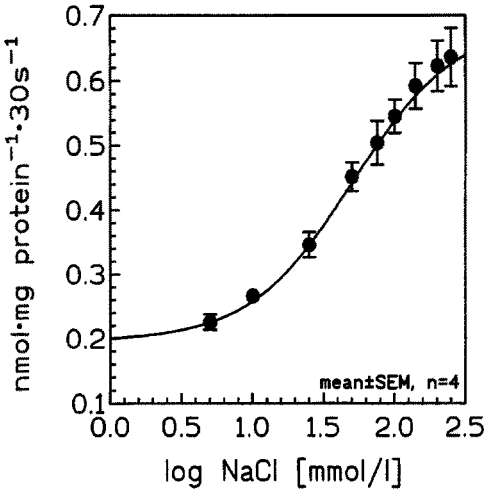


Fig. 4 Uptake of alanine into BBMV as a function of external NaCl concentration

BBMV were preloaded with 20 mmol/l HEPES/Tris buffer, 280 mmol/l mannitol and 0.25 mmol/l $CaCl_2$, pH 7.5, were incubated for 30 s of incubation in medium comprised of 20 mmol/l HEPES/Tris buffer pH 7.5, 0.25 mmol/l $CaCl_2$, 0.1 mmol/l alanine and 0 to 150 mmol/l NaCl replacing isoosmotically mannitol. Uptake rates were submitted to non-linear regression analysis based on competition curve with one component allowing the Hill coefficient to be determined as 1.13 ± 0.12. Data shown represent mean ± SEM of 3 preparations in triplicate.

Kinetics of alanine transport into BBMV as a function of NaCl concentration

For determination of the flux coupling ratio of Na^+/alanine cotransport, BBMV were incubated in the presence of increasing concentrations of NaCl replacing isoosmotically mannitol in the medium. Initial rate of uptake of 0.1 mmol/l ^{3}H-alanine increased in a sigmoid fashion as a function of log external [NaCl] as shown in Fig. 4. Uptake data were subjected to non-linear regression analysis based on a competition curve with one component. The Hill-coefficient derived was 1.13 ± 0.12 and the Na+ concentration causing half maximal stimulation of uptake was found to be 51.4 ± 1.3 mmol/l. Alanine influx therefore appears to be the consequence of Na^+/alanine cotransport operating with a 1:1 stoichiometry.

Effects of amino acid modifying agents on the alanine transport systems

The effects of the various group specific reagents were investigated with respect to inhibition of both, the Na^+ dependent and Na^+ independent transport pathways. BBMV, preincubated with the modifying compounds were washed free of the agents and uptake of 0.1 mmol/l ^{3}H-alanine was determined at 1 min, 5 min and 60 min of incubation. Concentrations of up to $5 \cdot 10^{-3}$ mol/l of the agents were used to modify the transporters. Fig. 5 shows the corresponding uptake rates after treatment of BBMV with $2 \cdot 10^{-3}$ mol/l of the compounds. Only agents which react selectively with SH-groups (NEM, PCMBS and DTNB) or NH_2-groups (PITC) were found to inhibit significantly Na^+ dependent and Na^+ independent alanine influx into BBMV. The most potent compound identified was PCMBS. However, PCMBS at a concentration of 2 mmol/l did not only inhibit influx of alanine into BBMV but also equilibrium uptake, indicating that the size and/or vesicle integrity was also dramatically affected. The dose dependence of the PCMBS action on Na^+ dependent alanine influx revealed an EC_{50} value of 450 ± 48 µmol/l (Fig. 6a). For this reason all further experiments employing PCMBS were performed at a concentration of

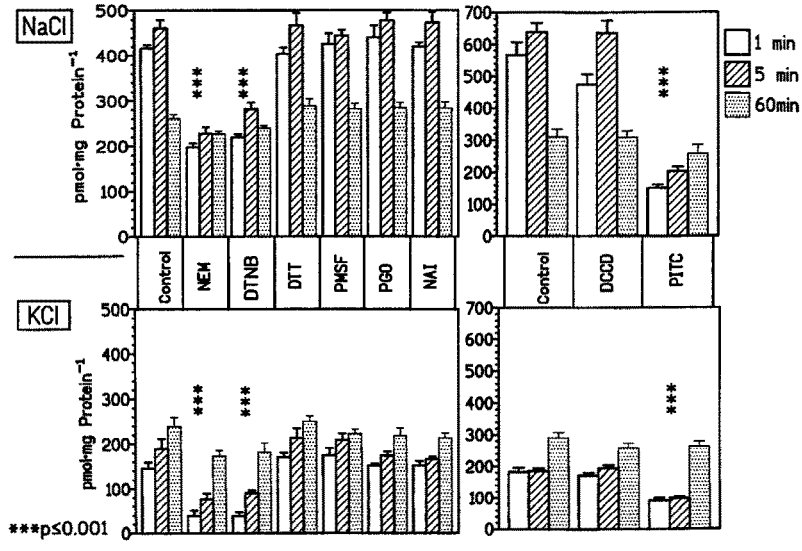


Fig. 5 Effect of the various group specific reagents on Na^+ dependent and Na^+ independent alanine transport into BBMV

BBMV were treated as described in the methods section with 2 mmol/l of the different group specific reagents and uptake rates were determined at 1 min, 5 min and 60 min of incubation in medium containing 20 mmol/l HEPES/Tris buffer 7.5, 80 mmol/l mannitol, 0.25 mmol/l $CaCl_2$, 0.1 mmol/l alanine and either 100 mmol/l NaCl or 100 mmol/l KCl. Data shown represent mean ± SEM of 3 preparations in triplicate.

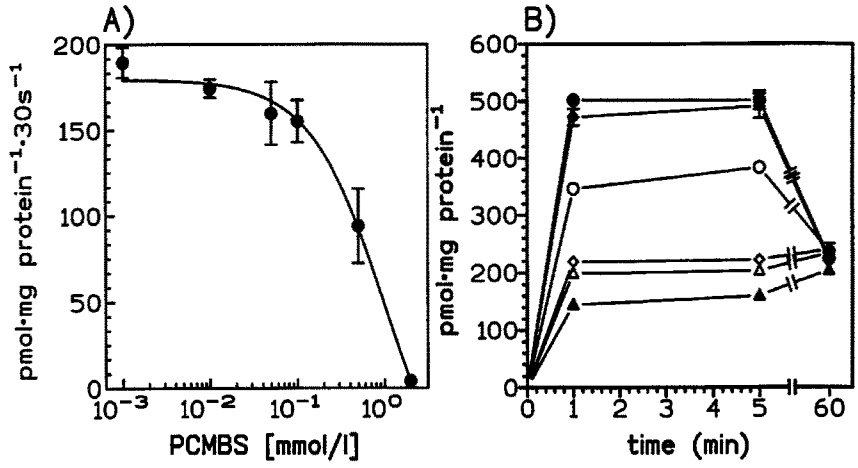


Fig. 6 Effect of PCMBS treatment of BBMV on Na^+ dependent uptake of alanine into BBMV and reversibility of PCMBS action by DTT

Fig. 6a: Dose dependence of inhibition of Na^+ dependent alanine influx by PCMBS:

BBMV loaded with 20 mmol/l HEPES/Tris buffer, 280 mmol/l mannitol, 0.25 mmol/l $CaCl_2$, pH 7.5, were treated with increasing concentrations of PCMBS as described in the method section and alanine influx was determined after 10 s of incubation in a medium composed of 20 mmol/l HEPES/Tris buffer pH 7.5, 80 mmol/l mannitol, 0.25 mmol/l $CaCl_2$, 100 mmol/l NaCl and 0.1 mmol/l alanine. Uptake rates were submitted to non-linear regression analysis based on a competition curve with one component and the corresponding EC_{50} value was determined as 480 ± 48 µmol/l (r^2 = 0.843).

Fig. 6b: Effect of DTT on PCMBS induced inhibition of alanine uptake:

Control BBMV or PCMBS (500 µmol/l) treated BBMV were preincubated with or without 2 mmol/l DTT as described in the method section and Na^+ dependent and Na^+ independent alanine uptake (0.1 mmol/l) was determined as described above. Uptake into control vesicles treated with 2 mmol/l DTT (100 mmol/l NaCl ●, 100 mmol/l KCl ✧) or BBMV treated with PCMBS (100 mmol/l NaCl ○, 100 mmol/l KCl ▲) or PCMBS followed by 2 mmol/l DTT (100 mmol/l NaCl ✦, 100 mmol/l KCl Δ) is plotted as a function of time as the mean ± SEM of 2 preparations in duplicate.

Fig. 7 Effect of PCMBS treatment on initial rate uptake of alanine into BBMV as a function of substrate concentration in the presence of an inwardly directed NaCl or KCl gradient

BBMV were preloaded with a buffer containing 20 mmol/l HEPES/Tris, 280 mmol/l mannitol and 0.25 mmol/l $CaCl_2$, pH 7.5. Uptake was measured for 10 s of incubation in a medium consisting of 20 mmol/l HEPES/Tris buffer pH 7.5, 80 mmol/l mannitol, 0.25 mmol/l $CaCl_2$, 100 mmol/l NaCl or 100 mmol/l KCl and 0.1 mmol/l to 5 mmol/l alanine. Uptake rates measured in control vesicles (NaCl ●, KCl ○) or vesicles treated with 500 µmol/l PCMBS (NaCl ■, KCl □) as described in the methods section were submitted to non-linear regression analysis based on a Michaelis Menten equation including a linear term and kinetic constants were derived from the corresponding Eadie Hofstee plots. Data shown represent mean ± SEM of 2 preparations in triplicate.

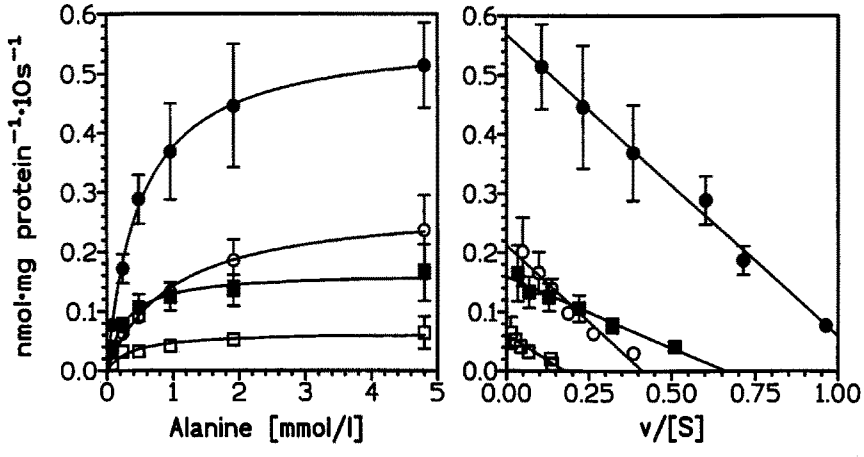


Table 1 Unidirectional influx of glutamate into placental BBMV treated with different group specific reagents

Control	NaCl-gradient (0.78 ± 0.12 nmol·mg protein^{-1}·30s^{-1}) 100 %	KCl-gradient (0.18 ± 0.05 nmol·mg protein^{-1}·30s^{-1}) 100 %
NEM	82.8 ± 10.1	94.2 ± 8.3
DTNB	104.1 ± 8.1	92.1 ± 5.6
PCMBS	90.3 ± 5.9	98.1 ± 10.4
DTT	103.4 ± 13.0	105.9 ± 7.9
PMSF	87.8 ± 11.1	97.8 ± 4.9
DCCD	42.3 ± 15.3***	88.1 ± 9.9
PITC	53.9 ± 16.6***	78.2 ± 12.1

*** $p \leq 0.001$

BBMV were preloaded with a buffer containing 20 mmol/l HEPES/Tris, 80 mmol/l mannitol, 100 mmol/l KCl and 0.25 mmol/l $CaCl_2$, pH 7.5. Membrane vesicles were treated with 2 mmol/l of the different group specific reagents as described in the methods section and uptake of 0.1 mmol/l glutamate was measured after 30 s of incubation in a medium consisting of 20 mmol/l HEPES/Tris buffer pH 7.5, 80 mmol/l mannitol, 0.25 mmol/l $CaCl_2$, 100 mmol/l NaCl or 100 mmol/l KCl. Data represent mean ± SEM of 2 membrane preparations in triplicate.

500 µmol/l. Pretreatment of BBMV with this concentration of PCMBS was found to reduce alanine influx at initial rates without altering alanine concentration under equilibrium conditions. The specificity of the compounds employed with respect to their ability to alter alanine transport selectively was proven by measuring Na^+-dependent influx of ^{3}H-glutamate into BBMV treated as described before. As shown in Table 1, in contrast to the effects on alanine influx, NEM, PCMBS and DTNB did not alter Na^+-dependent glutamate transport into BBMV. Glutamate uptake instead was found to be reduced significantly only by DCCD and PITC. Therefore, alterations of alanine transport by the SH-modifying agents appears to be specific.

PCMBS effects on the kinetics of Na+ dependent alanine uptake and its reversibility

To study the impact of PCMBS treatment on the kinetics of alanine uptake into placental membrane vesicles, Na^+ dependent and Na^+ independent initial rate uptake was determined as a function of alanine concentration (0.1 to 5 mmol/l) in BBMV treated with 500 µmol/l PCMBS (Fig. 7). Kinetic analysis revealed that the V_{max} of the Na^+ dependent system was reduced significantly from 0.57 ± 0.06 to 0.16 ± 0.05 nmol·mg protein^{-1}·10s^{-1} whereas the K_m values remained unaffected (0.434 ± 0.08 mmol/l versus 0.415 ± 0.13 mmol/l). Similarly, the K_m values of the Na^+ independent system remained unaffected by the treatment (0.425 ± 0.064 mmol/l versus 0.390 ± 0.090 mmol/l) whereas the V_{max} value was reduced significantly ($p < 0.001$) by PCMBS preincubation (Fig. 7).

The organic mercury compound PCMBS directly interacts with free SH-groups to form a PCMBS/SH-group complex. Since the mercaptocompound dithiotreitol (DTT) has been shown to act as an antagonist for non-covalent bound PCMBS (4), we determined whether DTT can reverse PCMBS-mediated inhibition of alanine influx into the membrane vesicles.

BBMV pretreated with 500 µmol/l PCMBS were incubated with increasing concentrations of DTT (0.1 to 2 mmol/l) and uptake of 0.1 mmol/l ^{3}H-alanine was measured as a function of time in the presence of a NaCl gradient or KCl gradient. Control BBMV were treated with DTT only. As shown in Fig. 6b, 2 mmol/l DTT itself did not affect transport activity of Na^+ dependent alanine transport when compared with untreated BBMV. In PCMBS-treated vesicles alanine uptake was significantly ($p < 0.001$) reduced and was almost completely restored after preincubation of PCMBS-treated BBMV with 2 mmol/l DTT. Na^+ independent alanine transport also was reduced by 30 % after PCMBS-treatment and restored by DTT.

To determine whether the free SH-groups identified as essential for transport function are located within the substrate binding site of the alanine transporter, we have performed substrate protection experiments. BBMV were incubated for 30 min in the presence of 2 mmol/l of either alanine or leucine followed by treatment with 500 µmol/l PCMBS as described above. BBMV were then washed twice with an excess of loading buffer (20 mmol/l HEPES/Tris buffer, 280 mmol/l mannitol, 0.25 mmol/l $CaCl_2$, pH 7.5) and pelleted at 35.000 x g (30 min). Uptake studies using 0.1 mmol/l alanine were carried out in the presence of a Na^+ gradient or the presence of a K^+ gradient, resp. BBMV preincubated only with alanine or leucine served as a control. As shown in Table 2, transport activity was fully restored in vesicles when substrates were washed out. However, when protected BBMV were treated with PCMBS neither substrate prevented the transporter of beeing blocked by PCMBS. This result indicates that the free SH-groups modified by PCMBS are most likely not located within the substrate binding site of the transport protein.

Table 2 Effect of PCMBS on alanine transport into BBMV in the absence or the presence of alanine or leucine

	NaCl-gradient	KCl-gradient
	uptake (pmol·mg protein^{-1})	
incubation time		
Control		
1 min	520.4 ± 50.8	161.2 ± 12.3
5 min	622.8 ± 45.9	144.2 ± 14.4
60 min	280.3 ± 23.9	298.5 ± 22.4
preincubation with 2 mmol/l alanine		
1 min	534.8 ± 34.5	174.4 ± 22.6
5 min	644.2 ± 33.9	186.2 ± 20.1
60 min	307.2 ± 33.2	310.1 ± 25.0
preincubation with 2 mmol/l leucine		
1 min	502.1 ± 29.8	143.7 ± 22.3
5 min	567.3 ± 42.3	164.1 ± 24.4
60 min	283.6 ± 23.7	292.4 ± 31.8
preincubation with 500 µmol/l PCMBS		
1 min	198.2 ± 25.3***	60.9 ± 11.7***
5 min	187.1 ± 42.2***	99.3 ± 18.6***
60 min	265.3 ± 32.4	277.5 ± 27.4
preincubation with alanine and PCMBS		
1 min	177.4 ± 35.9***	55.1 ± 13.2***
5 min	190.0 ± 37.5***	112.0 ± 19.0***
60 min	271.1 ± 28.6	288.5 ± 23.4
preincubation with leucine and PCMBS		
1 min	165.2 ± 26.7***	56.9 ± 19.9***
5 min	178.3 ± 32.1***	89.4 ± 17.0***
60 min	280.3 ± 23.9	265.7 ± 22.0

*** $p \leq 0.001$

BBMV were preloaded with a buffer containing 20 mmol/l HEPES/Tris, pH 7.5, 280 mmol/l mannitol and 0.25 mmol/l $CaCl_2$. Membrane vesicles were treated with 2 mmol/l of the different amino acids either in the absence or the presence of 500 µmol/l PCMBS as described in the methods section. Uptake of 0.25 mmol/l alanine was determined after 1 min, 5 min and 60 min of incubation in a medium consisting of 20 mmol/l HEPES/Tris buffer pH 7.5, 80 mmol/l mannitol, 0.25 mmol/l $CaCl_2$, 100 mmol/l NaCl or 100 mmol/l KCl. Data represent mean ± SEM of 3 membrane preparations in triplicate.

Discussion

Basic characteristics of Na+ dependent and Na+ independent alanine transport

A number of studies demonstrated that both Na^+ dependent and Na^+ independent systems mediate amino acid transport across the human placental microvillous membrane. With respect to neutral amino acids, similar transport activity has been shown in placental BBMV (1, 8, 11). In the present study the two systems mediating alanine influx into BBMV could be separated kinetically and by inhibition studies using selected amino acids with preference for either system. The Na^+ dependent and Na^+ independent transporters for alanine have almost identical affinity constants but different maximal transport capacities. As shown for the first time, Na^+ dependent alanine flux occurs with a 1:1 coupling ratio and the presence of an inwardly directed Na^+ gradient increases the V_{max} of the system about two times with a half maximal effect at an external NaCl concentration of 52 mmol/l.

The Na^+ dependent pathway clearly prefers alanine, glutamine, meAIB and methionine but is not shared by the more hydrophobic amino acids leucine, trytophan and tyrosine. The Na^+ independent uptake route appears to be shared by alanine, leucine, tyrosine, glutamine and methionine but not by meAIB. Charged amino acids (lysine, glutamate) and proline did not interact with either system. The demonstrated substrate specificities for the two systems are consistent with previous observations concerning the lack of inhibition of Na^+ dependent alanine uptake by meAIB and the inhibition of Na^+ independent flux by leucine (11). However, in the present study no inhibition of Na^+ dependent flux was found with leucine or proline, whereas 20 mmol/l leucine and proline inhibited Na^+/alanine cotransport (0.1 mmol/l alanine) by 54 % and 83 % in a previous report (11). Leucine and meAIB therefore clearly can be used to separate the Na^+ dependent system from the Na^+ independent transport pathway. That the two transporters indeed represent two distinct membrane proteins is supported by the demonstration of the distinct substrate specificities together with the observation that the presence or the absence of Na^+ ions failed to affect the substrate affinity for transport.

Effects of group specific agents

In order to identify functional groups essential for transport activity within the two transport proteins we employed a variety of agents known to react specifically with amino acid side chains or particular groups in membrane proteins. Specificity of action of the side specific compounds with the Na^+ dependent and Na^+ independent alanine transporters was established by comparing the effects with those on electrogenic Na^+-dependent glutamate transport.

Besides the NH_2-reactive agent PITC, only compounds reacting with free SH-groups (DTNB, NEM, PCMBS) were found to affect significantly alanine transport function. In contrast to alanine flux, Na^+ dependent glutamate transport was found to be altered only by PITC and DCCD but not by agents modifying SH-groups. Alterations in alanine transport by agents interacting with sulfhydryl groups are therefore specific and are not a consequence of altered BBMV integrity and stability. Inhibition of alanine transport by compounds reacting with free SH-groups has also been shown in isolated intestinal brush border membrane vesicles (12).

Since the compounds reacting with vicinal thiol groups (PAO) and disulfide bonds (DTT) failed to alter the activity of the alanine transporters, only the free thiol groups appear to be essential for transport function. Modifications of thiol groups in proteins by DTNB, NEM and PCMBS occur generally by different mechanisms: NEM

is a cyclic maleimide derivative with a *cis*-douple bond, which interacts with free SH-groups by a nucleophilic covalent addition reaction (4). For this reason NEM transport inhibition is usually reversible. The covalent non-penetrating SH-reagent DTNB has a disulfide group that has a high standard oxidation-reduction potential which reacts with free SH-groups by a disulfide-SH-group exchange reaction (4). The mercurial compound PCMBS on the other hand interacts with free SH-groups to form a PCMBS/SH-group complex which is reversed by mercaptocompounds like dithiotreitol (DTT) and 2-mercaptoethanol (3). Since the water soluble PCMBS was found to be the most potent inhibitor of alanine transport its interaction with SH-groups was studied further with respect to reversibility and site of action.

The reverse reaction by DTT on the complex of PCMBS/SH-group is chemically defined as a double disulfide exchange reaction (4) and by the same type of reaction DTT reduces the cysteine-cysteine bridges. In the present study DTT was found to be a potent antagonist for non-covalent bound PCMBS, able to fully restore PCMBS mediated inhibition of both the Na^+ dependent and Na^+ independent alanine transporters. It was obvious that DTT did not affect alanine transport by opening cysteine S-S groups in control BBMV but efficiently reactivated originally free SH-groups after exposure to PCMBS. Blocking of the free SH-groups caused a significant reduction of the V_{max} of both, the Na^+ dependent transporter from 0.57 ± 0.06 to 0.16 ± 0.05 nmol·mg protein^{-1}·10s^{-1} and of the Na^+ independent transporter from 0.21 ± 0.08 to 0.07 ± 0.03 nmol·mg protein^{-1}·10s^{-1}. The corresponding K_m values were not affected significantly. The fact that substrate affinity was not altered suggested already that the functional groups modified by PCMBS are not part of the substrate binding site. This was further substantiated by protection experiments with alanine serving as a substrate binding site blocker for both systems and leucine as a specific blocker for the Na^+ independent transporter. Since both substrates failed to protect the transporters of being modified, the free SH-groups appear not to be located in or near the substrate binding pocket. Although the SH groups are not involved in substrate binding, the groups are important for maximal transport velocity and should be kept in a reduced state. Since free SH-groups are also targets of transition state metals such as cadmium, the SH-groups identified in plazental transporters for neutral amino acids could be of particular importance for fetal supply of amino acids in conditions of maternal heavy metal incorporation.

In conclusion, we have demonstrated for the first time, that the two membrane proteins responsible for transport of several neutral amino acids across the human placental brush border membrane contain free SH-groups which are important for transport function. These groups are not located within the substrate binding site but they are essential for maximal transport activity.

References

1. Alonso-Torre SR, Serrano M, Medina J, Alvarado F (1992) Heterogeneity of L-alanine transport systems in brush-border membrane vesicles from rat placenta during late gestation. Biochem J 288:47–53
2. Battaglia FC (1989) An update of fetal and placental metabolism: carbohydrate and amino acids. Biol Neonate 55:347–354
3. Blumrich M, Petzinger E (1990) Membrane transport of conjugated and unconjugated bile acids into hepatocytes is susceptible to SH-blocking reagents. Biochim Biophys Acta 1029:1–12
4. Blumrich M, Petzinger E (1993) Two distinct types of SH-groups are necessary for bumetanide and bile acid uptake into isolated rat hepatocytes. Biochim Biophys Acta 1149:278–284
5. Carter BS, Moores RR, Battaglia FC (1991) Placental transport and fetal and placental metabolism of amino acids. J Nutr Biochem 2:4–13
6. Ganapathy V, Balkovetz DF, Ganapathy ME, Mahesh VB, Devoe LD, Leibach FH (1987) Evidence for histidyl and carboxy groups at the active site of the human placental Na^+-H^+ exchanger. Biochem J 254:473–477
7. Ganapathy ME, Howard JC, Devoe LD, Ganapathy V (1986) Characterisation of tryptophan transport in human placental brush-border vesicles. Biochem J 238:201–208
8. Hoeltzli SD, Smith CH (1989) Alanine transport systems in isolated basal plasma membrane of human placenta. Am J Physiol 256:C630–C637
9. Iioka H, Hisanaga H, Moriyama IS, Akada S, Shimamoto T, Yamada Y, Ichijo M (1990) Characterisation of human placental activity for transport of L-alanine, using brush border (microvillous) membrane vesicles. Placenta 13:179–190
10. Iioka H, Moriyama IS, Kyuma M, Itoh K, Amasaki M, Ichijo M (1985) Studies on L-glutamate transport mechanism in human placental trophoblast microvilli membrane. Acta Obst Gynaec Japan 37:207–212
11. Johnsen LW, Smith CH (1988) Neutral amino acid transport systems of microvillous membrane of human placenta. Am J Physiol 254:C773–C780
12. Kramer W, Dürckheimer W, Girbig F, Gutjahr U, Leipe I, Oekonomopolos R (1990) Influence of amino acid side-chain modification on the uptake system for β lactam antibiotics and dipeptides from rabbit small intestine. Biochim Biophys Acta 1028:174–182
13. Kudo Y, Yamada K, Fujiwara A, Kawasaki T (1987) Characterisation of amino acid transport systems in human placental brush-border vesicles. Biochim Biophys Acta 904:309–318
14. Miyamoto Y, Tiruppathi C, Ganapathy V, Leibach FH (1989) Involvement of thiol groups in the function of the dipeptide/proton cotransport system in rabbit renal brush-border membrane vesicles. Biochim Biophys Acta 978:25–31
15. Morriss FH, Boyd DH (1988) Placental transport. In: Knobil E, Neill J (eds) The physiology of reproduction. Raven Press, New York, pp 2043–2083
16. Smith CH, Moe AJ, Ganapathy V (1992) Nutrient transport pathways across the epithelium of the placenta. Annu Rev Nut 12:183–206
17. Yudilevich DL, Barros LF (1990) Transport of amino acids and nucleosides in the placenta. Biochem Society Transactions 18:1136–1140
18. Yudilevich DL, Sweiry JH (1985) Transport of amino acids in the placenta. Biochim Biophys Acta 822:169–201