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Functional Characteristics of Neutral Amino Acid Transporter in Opossum Kidney (OK) Cells

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The characteristics of Na^+-dependent cycloleucine uptake was investigated in OK cells with regard to substrate specificity and regulation by protein kinase C (PKC). Inhibition studies with different synthetic and natural amino acids showed a broad spectrum affinity to neutral amino acids regardless of their different side chains including branched or aromatic, indicating that the Na^+-dependent cycloleucine uptake in OK cells is mediated by System B^0 or System B^0-like transporter rather than the classical System A or ASC. Phorbol 12-myristate 13-acetate (PMA) and phorbol 12,13-dibutyrate, but not 4 α-PMA elicited a time-dependent biphasic stimulation of Na^+-dependent cycloleucine uptake, which produced early transient peak at 30 min and late sustained peak at 180 min. Both the early and late stimulations by PMA were due to an increase in Vmax and not due to a change in Km. PKC inhibitors blocked both the early and late stimulation by PMA, while protein synthesis inhibitors blocked the late stimulation only. These results suggest the existence and regulation by PKC of System B^0 or System B^0-like broad spectrum transport system for neutral amino acids in OK cells.

Key Words : Neutral amino acid transport, Opossum kidney cells, System B^0, Phorbol ester, Protein kinase C

INTRODUCTION

In mammalian cells, a large number of distinct transport systems for amino acids are classified based on differences in substrate specificity, kinetic properties, and ionic dependence (Christensen, 1990; Kilberg et al, 1993; McGivan & Pastor-Anglada, 1994; Mailliard et al, 1995). Although the degree of involvement of one transport system in the uptake of amino acids may differ from tissue to tissue, but several amino acid transport systems are known to be expressed constitutively and ubiquitously. A, ASC, and L are such classical systems which are responsible for the bulk of the cellular uptake of neutral amino acids in most types of mammalian cells.

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System A, which was originally designated for alanine, is Na^+-dependent and potently transports neutral amino acids with short, polar, or linear side chains. These include synthetic model amino acid AIB and N-methylated derivatives such as MeAIB. System ASC was originally designated for alanine, serine and cysteine. It is also Na^+-dependent, but excludes MeAIB. System L is a Na^+-independent system which shows high affinity to leucine.

In kidney tubules, filtered amino acids are reabsorbed via luminal uptake and peritubular exit. Luminal uptake plays also a nutritive role to provide substrates for protein synthesis, gluconeogenesis, ammoniagenesis, glutamine systhesis and arginine formation. In the cells of late proximal and postproximal nephron where luminal delivery of amino acids is very small, peritubular uptake plays an important role. Although facilitated diffusion through basolateral membrane is believed to be a major pathway for both peritubular exit and uptake, involvement of some Na^+-dependent systems including A and ASC systems has been implicated (Silbernagl, 1988; Christensen,

1990).

With regard to luminal uptake, it is now believed that a Na^+-dependent broad spectrum transporter System B^o plays a major role. System B^o was first described by Lynch & McGivan (1987) in bovine renal brush border membrane. The term B indicates the broad substrate specificity, and the superscript indicates preference for neutral amino acids. System B^o is differentiated from system $B^{o,+}$ which was first described in mouse blastocyst (Van Winkle et al, 1985) and accepts cationic as well as neutral amino acids. It seems that system B^o is identical or closely related with System B which was described in intestinal epithelium (Mailliard et al, 1995).

OK cells, an established cell line derived from the American opossum kidney (Koyama et al, 1978), have characteristics of renal proximal tubules (Malmstrom et al, 1987). In the preliminary studies on amino acid transport in kidney cells, we observed that a neutral non-metabolizable model amino acid cycloleucine is accumulated into OK cells through System B^o-like mechanism. This study thus was carried out to investigate further the characteristics and regulation of the cycloleucine transport system in these cells.

METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM), Dulbecco's phosphate buffered saline (PBS), trypsin, penicillin, streptomycin and fetal bovine serum were obtained from GIBCO/BRL (Grand Island, NY). Amino acids, 1-aminocyclopentane-1-carboxylic acid (cycloleucine), 2-aminoisobutyric acid (AIB), 2-(methylamino)isobutyric acid (MeAIB) and 2-aminoendobicyclo[2,2,1]heptane-2-carboxylic acid (BCH) were purchased from Sigma (St. Louis, MO). [^{14}C]-cycloleucine was purchased from Amersham (Arlington Heights, IL). Phorbol 12-myristate 13-acetate (PMA), 4 α-PMA, phorbol 12,13-dibutyrate (PDBu), staurosporine and D-erythrosphingosine were purchased from RBI (Natick, MA), and cycloheximide and actinomycin-D were obtained from Calbiochem (La Jolla, CA).

Cell culture

OK cells obtained from American Type Culture Collection (Rockville, MD) were routinely maintained on plastic culture flasks in DMEM supplemented with 0.584 g/L glutamine, 10% fetal bovine serum, 100 IU/ml penicillin and 100 μg/ml streptomycin. Cells were trypsinized when cells became confluent (approximately every 4~5 days) using 0.05% trypsin/0.53 mM EDTA solution and reseeded at one-eighth the original density. For uptake experiments, cells were subcultured on 24-well plastic culture plates at a density of 10^5 cells/well. The cells were fed with fresh media every other day. The cells on 24-well plates became confluent after 3-4 days of post-seeding under microscopic observation. After 6 days of post-seeding, total cell counts and protein contents were maintained at a steady level. Uptake was measured in cells 8-10 days of post-seeding. The cells used for this study were between passage of 67 and 82.

Measurements of [^{14}C]-cycloleucine uptake

[^{14}C]-cycloleucine uptake was measured in PBS at 37°C under atmospheric air. Cells on 24-well plates were washed twice with prewarmed PBS to remove the culture medium and serum. After equilibration in PBS for 2 hrs, cells were exposed to 100 μM [^{14}C]-cycloleucine in 0.5 ml PBS and incubated for a given time period. During incubation, the plates were shaked at 70-80/min. Incubation was stopped by rapid removal of the incubation medium and washing the cells 3 times with cold PBS. Cells on the well plates were then dissolved in 0.5 ml of 0.5% Triton X-100. The cell extracts were transferred into scintillation vials containing 10 ml of scintillation fluid and radioactivity was determined. [^{14}C]-cycloleucine uptake/well was expressed as nmolar uptake/mg protein. Protein concentration was determined according to Bradford (1976) with bovine serum albumin as a standard.

Data analysis

Data were expressed as mean±S.E. of more than 4 determinations in cells of at least 2 different passages. When needed, the data were analyzed by one

way analysis of variance followed by the unpaired t-test. A value of $p<0.05$ was considered statistically significant. Transport kinetic parameters were obtained by fitting data to the Michaelis-Menten equation by nonlinear least square fitting program (Enzfit, Biosoft).

RESULTS

Time course of [^{14}C]-cycloleucine uptake

Uptake of [^{14}C]-cycloleucine was linear up to 60 min at concentration of 100 μM in either NaCl or Na^+-free tetramethylammonium chloride (TMA-Cl) medium. Na^+-dependent component of total [^{14}C]-cycloleucine uptake, which was obtained by subtracting the uptake measured in TMA-Cl medium from the total uptake in NaCl medium, was about 50% of the total uptake. The uptake of [^{14}C]-cycloleucine measured in the presence of 20 mM cold cycloleucine was less than 5% of the total uptake (Fig. 1).

Interaction with AIB, MeAIB and BCH

In Fig. 2, we determined the effect of 3 different synthetic amino acids on Na^+-dependent [^{14}C]-cycloleucine uptake. They have been frequently used to distinguish the classical A, ASC and L neutral amino acid transport systems. AIB inhibited Na^+-dependent [^{14}C]-cycloleucine uptake at concentrations above 1 mM and produced 63% inhibition at 10 mM. MeAIB was completely ineffective in inhibiting the uptake. A bicyclo amino acid BCH significantly inhibited the

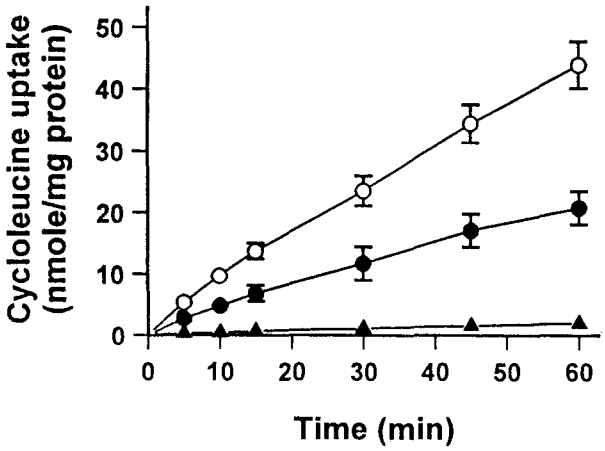

Fig. 1. Time course of cycloleucine uptake in OK cell monolayer. Monolayers of OK cells were incubated in Dulbecco's phosphate buffered saline (PBS) containing 100 μM [^{14}C]-cycloleucine in the presence(○) and absence(●) of Na^+. To estimate the uptake by simple diffusion, [^{14}C]-cycloleucine uptake was also determined in Na^+-free medium in the presence of 20 mM non-labeled cycloleucine(▲). For Na^+-depleted medium, NaCl and Na_2HPO_4 were replaced by TMA-Cl and K_2HPO_4, respectively. [^{14}C]-cycloleucine uptake was determined for designated time periods at 37°C. Incubation was stopped by rapid removal of the incubation medium and washing the monolayers 3 times with cold PBS. Each point is mean±S.E. of the values determined in 4 monolayers.

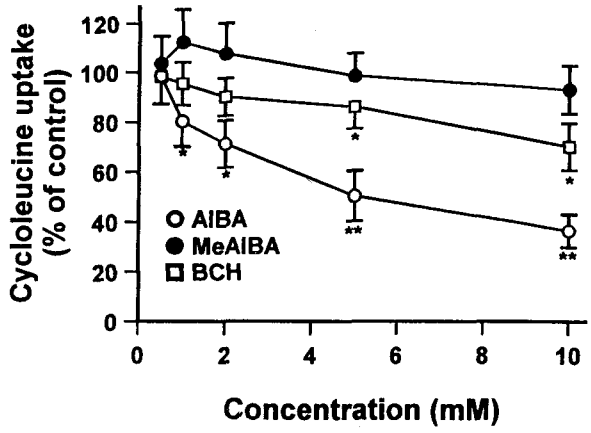

Fig. 2. Concentration-dependent effects of AIBA, MeAIBA and BCH on Na^+-dependent cycloleucine uptake in OK cell monolayer. [^{14}C]-cycloleucine uptake was determined for 10 min by incubating OK cell monolayers in Dulbecco's phosphate buffered saline (PBS) containing 100 μM [^{14}C]-cycloleucine in the presence of indicated concentrations of AIBA (○), MeAIBA (●) or BCH (□). Na^+-dependent uptake was obtained by subtracting the uptake in the absence of Na^+ from the corresponding uptake in the presence of Na^+. For Na^+-depleted medium, NaCl and Na_2HPO_4 were replaced by TMA-Cl and K_2HPO_4, respectively. Incubation was stopped by rapid removal of the incubation medium and washing the monolayers 3 times with cold PBS. Each point is mean ±S.E. of the value determined in 5 monolayers.
*,** Significantly different from the control ($p<0.05$, $p<0.01$).

Table 1. Effect of Various Amino Acids (5 mM) on Na^+-dependent ^{14}C-cycloleucine Uptake in OK Cell Monolayer

	Uptake (nmole/mg protein/10 min)	% inhibition
Control	4.86±0.67	
(Neutral nonpolar)		
Ala	0.17±0.04	96.5
Isoleu	1.10±0.18	77.4
Leu	0.26±0.04	94.7
Met	0.16±0.02	96.7
Phe	1.71±0.31	64.8
Pro	0.49±0.06	89.9
Trp	0.51±0.11	89.5
Val	0.97±0.17	80.0
(Neutral polar)		
Asn	0.14±0.02	97.1
Cys	0.15±0.02	96.9
Gln	0.51±0.08	89.5
Gly	0.96±0.19	80.2
Ser	0.17±0.03	96.5
Thr	0.48±0.07	90.1
(Basic)		
Arg	3.29±0.42**	32.3
His	1.13±0.17**	76.7
Lys	3.73±0.47*	23.3
(Acidic)		
Asp	4.64±0.66NS	4.5
Glu	4.71±0.81NS	3.1

Mean ±S.E. (n=4).
*,** Significantly different from the control ($p<0.05$, $p<0.01$).
NS nonsignificant.

uptake at concentrations of 5 and 10 mM.

Inhibition by amino acid analogues

Table 1 shows Na^+-dependent uptake of 100 μM [^{14}C]-cycloleucine in the presence of different unlabelled amino acid analogues (5 mM). The uptake was strongly inhibited by both polar and nonpolar neutral amino acids regardless of their side chains including branched or aromatic. Among basic amino acids, lysine and arginine showed weak inhibition. In contrast, histidine inhibited the uptake by 77%. Histidine has a relatively lower isoelectric point when compared with other basic amino acids and the charge of imidazole group is easily affected by the surrounding environment. When lowering the medium pH from 7.4 to 6.5, the magnitude of the inhibition by histidine

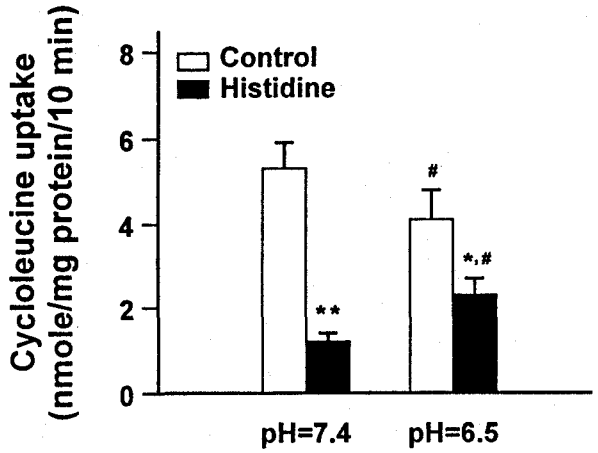

Fig. 3. pH-dependent inhibition of Na^+-dependent cycloleucine uptake by 5 mM histidine in OK cell monolayer. Na^+-dependent [^{14}C]-cycloleucine uptake was determined for 10 min in the presence and absence of 5 mM histidine at pH=7.4 and 6.5. Each point represents mean±S.E. of the value determined in 4 monolayers.
*,** Significantly different from the control($p<0.05$, $p<0.01$).
Significantly different from the respective value at pH 7.4 ($p<0.05$).

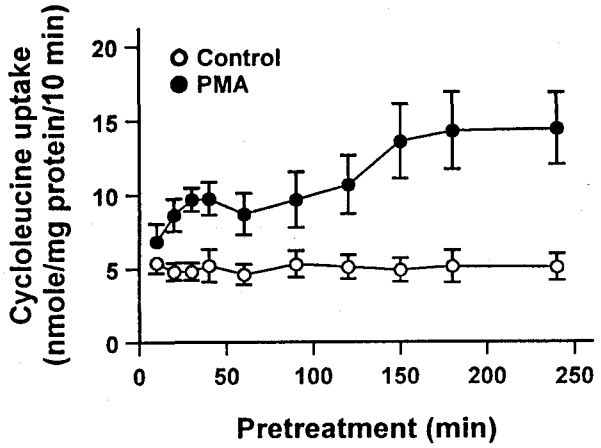

Fig. 4. Time-dependent effect of PMA on Na^+-dependent cycloleucine uptake in OK cell monolayer. Monolayers of OK cells were pretreated in Dulbecco's phosphate buffered saline (PBS) in the absence (○) and presence (●) of 10^{-6} M of PMA for designated time period and Na^+-dependent [^{14}C]-cycloleucine uptake was determined for 10 min. Each point is mean±S.E. of the value determined in 5 monolayers.

was attenuated to 44% (Fig. 3). These results suggest that the inhibition of the uptake by histidine at pH 7.4 is mostly mediated by the uncharged histidine molecule. Acidic amino acids, asparate and glutamate failed to inhibit the uptake.

Phorbol ester-induced stimulation

After equilibration in PBS for 2 hrs, cells were exposed to 10^{-6} M PMA for various time periods and Na^+-dependent [^{14}C]-cycloleucine uptake was determined. PMA stimulated the uptake in a biphasic manner depending on pretreatment time, resulting in two peaks. The first peak was usually observed at 30 min of pretreatment and showed 2-fold stimulation. After a slight decrease or plateau following the initial peak, PMA caused a second phase stimulation. This late stimulation usually appeared at about 90 min of pretreatment and reached its sustained peak after 180 min which showed 2.8-fold stimulation (Fig. 4). The results depicted in fig. 5 indicate that the pretreatment of PMA for 30 min or 180 min stimulated the uptake dose-dependently. Similary, another phorbol ester PDBu stimulated the uptake, while 4 α-PMA did not. (Fig. 6). When cells were pretreated with PMA for 30 or 180 min in the presence of PKC inhibitors, staurosporine (Tamaoki et al, 1986) or D-erythrosphingo-

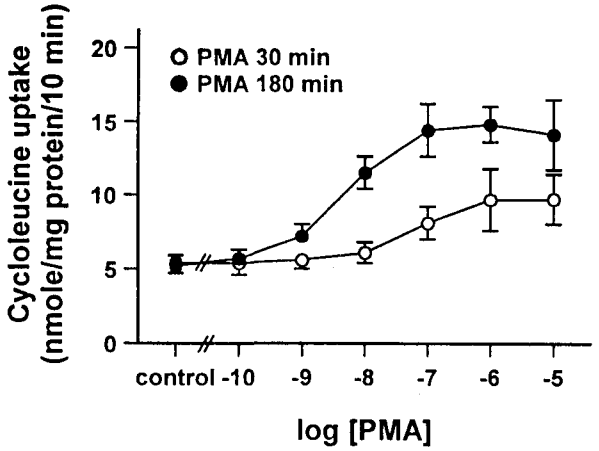

Fig. 5. Concentration-dependent effect of PMA on Na^+-dependent cycloleucine uptake in OK cell monolayer. Monolayers of OK cells were pretreated with indicated concentrations of PMA for 30 min (○) or 180 min (●) and Na^+-dependent [^{14}C]-cycloleucine uptake was determined for 10 min. Each point is mean ± S.E. of the value determined in 4 monolayers.

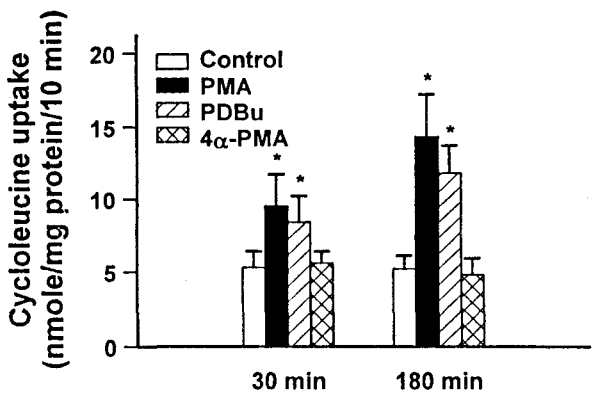

Fig. 6. Effects of PMA, PDBu and 4 α-PMA on Na^+-dependent cycloleucine uptake in OK cell monolayer. Monolayers of OK cells were pretreated with each 10^{-6} M of PMA, PDBu or 4 α-PMA for 30 min or 180 min, and Na^+-dependent [^{14}C]-cycloleucine uptake was determined for 10 min. Each point is mean±S.E. of the value determined in 4 monolayers.
*Significantly different from the respective control ($p< 0.01$).

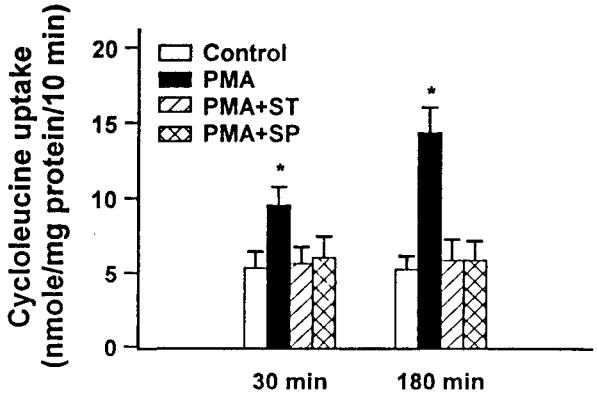

Fig. 7. Effects of protein kinase C inhibitors, staurosporine and D-erythrosphingosine, on PMA-induced stimulation of Na^+-dependent cycloleucine uptake in OK cell monolayer. Monolayers of OK cells were pretreated with 10^{-6} M of PMA in the presence and absence of staurosporine (ST, 100 nM) or D-erythrosphingosine (SP, 10 μ M) for 30 min or 180 min, and Na^+-dependent [^{14}C]-cycloleucine uptake was determined for 10 min. Each point is mean ± S.E. of the value determined in 6 monolayers.
*Significantly different from the respective control ($p< 0.01$).

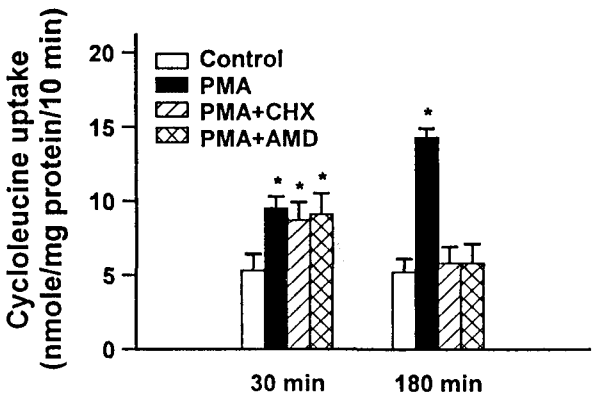

Fig. 8. Effects of cycloheximide and actinomycin-D on PMA-induced stimulation of Na^+-dependent cycloleucine uptake in OK cell monolayer. Monolayers of OK cells were pretreated with 10^{-6} M of PMA in the presence and absence of cycloheximide (CHX, 10 μg/ml) or actinomycin-D (AMD, 10 μg/ml) for 30 min or 180 min, and Na^+-dependent [^{14}C]-cycloleucine uptake was determined for 10 min. Each point is mean ± S.E. of the value determined in 6 monolayers.
*Significantly different from the respective control ($p < 0.01$).

Table 2. Kinetic parameters of Na^+-dependent cycloleucine uptake in OK cell monolayer

	Km (mM)	*Vmax* (nmole/mg protein/min)
Control	0.67 ± 0.08	3.19 ± 0.05
PMA-treated		
30 min	0.62 ± 0.05	5.13 ± 0.09
180 min	0.69 ± 0.10	6.47 ± 1.02

Mean ± S.E. (n=6).

sine (Hannun & Bell, 1989), the stimulatory effect of PMA was blocked (Fig. 7). The Na^+-dependent [^{14}C]-cycloleucine uptake was not affected by pretreatment of PKC inhibitors alone for 30 or 180 min (data not shown). These results indicate that the stimulation of the uptake by phorbol esters is mediated by activation of PKC. Nonlinear regression analysis of Na^+-dependent [^{14}C]-cycloleucine uptake showed a Km of 0.67 ± 0.08 mM and Vmax of 3.19 ± 0.05 nmole/ mg protein/ min. In cells pretreated with PMA for 30 and 180 min, the Vmax increased to 5.13 ± 0.09 and 6.47 ± 1.02 nmole/ mg protein/ min, respectively, while the Km remained unaffected significantly (Table 2).

Blocked PMA stimulation by cycloheximide and actinomycin-D

When cells were pretreated with PMA for 180 min in the presence of protein synthesis blockers, cycloheximide (Johnson et al, 1965) or actinomycin-D (White & Phillips, 1988), the stimulatory effect of PMA was completely abolished, while it remained unaffected with 30 min of pretreatment (Fig. 8).

DISCUSSION

OK cells, when grown as monolayer on plastic culture plates as well as permeable filters, are known to make functional tight junctions and exhibit vectorial transport which leads to formation of domes (States et al, 1991). In addition, it was demonstrated that uptake of uridine (Doherty & Jarvis, 1993) and hexose (Van den Bosch et al, 1989) in OK cell monolayer grown on plastic culture plates do reflect the properties of brush border membranes. Accordingly, [^{14}C]-cycloleucine uptake determined in the present study seems to reflect the transport through brush border membranes.

Inhibition studies with different synthetic and natural amino acid analogues indicate that Na^+-dependent [^{14}C]-cycloleucine uptake in OK cells is mediated by a broad spectrum system which is quite different from the classical A or ASC system. Na^+-dependent [^{14}C]-cycloleucine uptake was inhibited by AIB in a dose dependent manner, but MeAIB was completely ineffective in modifying the uptake. This distinguishes the uptake system from the System A, which is characteristically defined by exclusive uptake of, or inhibition by AIB or MeAIB (Christensen, 1990; Kilberg et al, 1993; McGivan & Pastor- Anglada, 1994; Mailliard et al, 1995). In addition, the inhibition of the uptake by BCH may exclude the involvement of the System ASC. BCH is known to be specifically transported through System L, a Na^+-independent neutral amino acid transporter. System A or ASC does not recognize BCH, while broad spectrum Systems B, B^o or $B^{o,+}$ have considerable affinity to BCH (Christensen, 1990; Kilberg et al, 1993; McGivan & Pastor-Anglada, 1994; Mailliard et al,

1995). These results, taken together with strong inhibition by most of the neutral amino acids regardless of their side chains, consistently suggest the involvement of a broad spectrum system.

Although the Na^+-dependent cycloleucine uptake was significantly inhibited by a basic amino acid histidine, the magnitude of inhibition was attenuated by lowering the medium pH. Histidine has a relatively lower isoelectric point compared with other basic amino acids and its electric charge is easily affected by its surrounding environment around physiological range of pH due to its imidazole group. Taken together, it is not likely that the inhibition of the uptake by basic amino acids reflects the system $B^{o,+}$ activity. Rather, it might be reasonable to assume that some uncharged fraction of basic amino acids at the experimental pH is responsible for the results. Accordingly, our findings are consistent with the suggestion that the Na^+-dependent [^{14}C]-cycloleucine uptake in OK cell is mediated by a System B^o or System B^o-like transporter.

System B^o was first described in bovine renal brush border membrane (Lynch & McGivan, 1987). The bovine renal epithelial cell line NBL-1 also expresses this system (Doyle & McGivan, 1992). Although it has been demonstrated that the LLC-PK1 (Sepulveda & Pearson, 1982) and MDCK (Boerner & Saier, 1982) cell lines express the classical System A, ASC and L activity, some evidences do not support the typical characteristics of these systems and rather imply the involvement of System B^o. Above considerations, taken together with our results, are consistent with the probability that the broad spectrum System B^o may be distributed widely in renal epithelial cells.

Although the proteins corresponding to these transport systems have not been identified, it is probable that System B from intestine and B^o from kidney are identical or closely related (McGivan & Pastor-Anglada, 1994). Recently, a cDNA was cloned from a human placental choriocarcinoma cell cDNA library which, when expressed in HeLa cells or Xenopus laevis oocytes, induces System B^o-like amino acid transport (Kekuda et al, 1996). The existence of a broad spectrum system for the reabsorption of amino acids in the brush border membrane of some epithelial cells can be rationalized physiologically as it allows the Na^+-gradient to drive the absorption of branched-chain and aromatic amino acids as well as aliphatic amino acids from the luminal fluid.

Several studies demonstrated the role of PKC in the regulation of amino acid transport (Amsler et al, 1983; Dawson & Cook 1987; Boerner & Saier, 1988; Pan & Stevens, 1995a; Pan & Stevens, 1995b). In the present study, the stimulatory effect of PMA on the Na^+-dependent [^{14}C]-cycloleucine uptake was clearly observed at concentrations as low as 10^{-9}, or 10^{-7} M with 180 or 30 min of pretreatment, respectively. The stimulation by PMA likely involves an activation of PKC, because it was blocked by PKC inhibitors, staurosporine (Tamaoki et al, 1986) or D-erythro-sphingosine (Hannun & Bell, 1989). Another phorbol ester PDBu also stimulated the uptake, but a non-active analogue of 4α-PMA did not. In a separate set of experiments (data not shown), forskolin (10 μM) or 8-Br-cAMP (100 μM) did not affect the Na^+-dependent [^{14}C]-cycloleucine uptake, indicating that protein kinase A is not likely to be involved in regulating the transport activity. Both the early and late phase stimulations were due to an increase in the transport capacity (Vmax), but not due to a change in transport affinity (Km). The late phase stimulation by PMA is likely to be involved a *de novo* protein synthesis. Transcription and/or translation events could be implicated because cycloheximide or actinomycin D blocked the stimulatory effect of PMA.

Although it is tempting to conclude that the change in transport capacity (Vmax) was due to increased copies of the carrier protein, we cannot rule out the possibility of a more complex scenario. Epithelial amino acid transport activities may be influenced by regulatory proteins or by the association of structural subunits within the membrane. It has been suggested that amino acid transport activity can be regulated by type II integral membrane glycoproteins with a single transmembrane-spanning domain (McGivan & Pastor-Anglada, 1994). Pathways involving such regulatory proteins may be an alternative means to regulate the amino acid transport activity.

Mechanism underlying the early phase stimulation is not clear at present. There are two potential sites for PKC-dependent phosphorylation in the System B^o-like transporter cloned from a human placental choriocarcinoma cell line (Kekuda et al, 1996). Phosphorylation of these sites may be a candidate to explain the short-term regulation by PKC. However, we can not preclude the possibility that PKC may exert its effect by affecting ionic gradients or mem-

brane potential as consequences of modification of ionic channels and/or pumps. More detailed and thorough investigation is needed to confirm the mechanism.

There are a number of genetic or acquired defects in amino acid transport. One of them is Hartnup disease in which there is a specific impairment of transport of neutral amino acids in the intestine and kidney (Christensen, 1990). Our results demonstrating the existence and regulation by PKC of System B^o or System B^o-like transport system for neutral amino acids in OK cells may provide a useful model for studying the physiology and pathophysiology of renal handling of neutral amino acids.

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