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Transport of 2-aminoisobutyric acid in cultured endothelial cells

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Abstract

Conflicting reports exist concerning the presence of a Na^+-coupled amino acid transport system in cultured endothelial cells. We have employed a non-metabolizable analog, 2-aminoisobutyric acid (AIB), to investigate the activity of Na^+-dependent amino acid transport in cultured human umbilical vein endothelial cells (HUVEC). We found a pronounced saturable, Na^+-dependent component of AIB uptake in 'fresh' (non-starved) HUVEC. The Na^+-dependent component accounted for 78% of total AIB uptake with a high sensitivity to external Na^+. The accumulation of AIB was inhibited by ouabain preincubation, consistent with the energetics of Na^+-coupled transport. Amiloride, an epithelial Na^+ channel blocker, also inhibited AIB transport at high concentration. The results strongly support the presence of a Na^+-coupled transport system of amino acid in HUVEC.

Key words: Sodium ion coupled transport; Endothelial cell; Amino acid analog; Ouabain; Amiloride

1. Introduction

Endothelial cells not only provide a selective barrier between blood and surrounding tissues but also serve as a metabolically active compartment for several vasoactive compounds [1–3]. Amino acids derived from surrounding media presumably plays a role in the regulation of the synthesis of these compounds. Yet only a few reports are available for the studying of the transport systems of amino acids in endothelial cells [4–7]. The activities for several Na^+-independent transport of amino acids are identified including those for large neutral amino acids, L-homocysteine, and L-cystine, among others; nevertheless, no evidence for the Na^+-dependent system A is reported [4–6]. A recent study, however, demonstrates that after amino acid starvation, a relatively high transport activity of the Na^+-dependent system A is observed in human umbilical vein endothelial cells where a specific substrate for system A, 2-methylaminoisobutyric acid (MeAIB), is employed [8]. Endothelial cells, like other cells, maintain Na^+ gradient across plasma membrane via Na^+/K^+ pump and $Na^+/K^+/Cl^-$ cotransport systems [9–12]. It is likely that the transport properties of Na^+-dependent system can be altered as the elements that maintain electrochemical gradient of Na^+, e.g., Na^+ pump activity, Na^+ leak permeability, membrane potential, and the activities of other Na^+-coupled transporter are altered. In this study, we examined the transport of 2-aminoisobutyric acid (AIB), a non-metabolizable model compound for the activity of Na^+-dependent system A [13,14], of cultured human umbilical vein endothelial cells and the effects of ouabain and amiloride, agents that are known to affect Na^+ pump and Na^+-H^+ exchanger, respectively.

2. Materials and methods

2.1. Endothelial cell culture

Human umbilical vein endothelial cells (HUVEC) were obtained and cultured as described previously [12,15]. Briefly, cells were grown in medium MCDB 107 containing 2% fetal bovine serum, epidermal growth factor (10 ng/ml), and a partially purified porcine brain-derived fibroblast growth factor (0.5 μg/ml) at 37°C. For transport studies, HUVEC between the 6th and 10th passages were removed by

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trypsinization and were subcultured onto 16 mm-diameter cell wells of disposable 24-well trays (Corning Glass, NY). They were examined for morphology and used at confluency.

2.2. AIB uptake measurements

The cells were washed twice in balanced salt solution (BSS) containing (in mM) 130.0 NaCl, 5.0 KCl, 2.0 $CaCl_2$, 1.0 $MgCl_2$, 20.0 Hepes with pH adjusted to 7.4 by Tris base. Preliminary experiments have demonstrated that incubation in BSS for a period over 6 h would not alter HUVEC viability, morphology, protein content, and K^+ content. For Na^+-free solution (Na^+-free BSS), NaCl was replaced by choline chloride. Initial rate of uptake of ^{14}C-AIB was determined by measuring cell-associated radioactivity after an appropriate incubation of HUVEC in BSS with ^{14}C-AIB added. First, cells were preicubated in BSS or Na^+-free BSS for 10 min. This period was extended for ouabain experiments to 1 h and would also include amiloride for some experiments. Cells were further washed twice with the respective solutions. Solutions containing ^{14}C-AIB was then replaced and cells incubated for the specified period of uptake time. After uptake period, cells were washed with 0.1 M $MgCl_2$ solution (containing 10 mM Hepes, pH 7.4) six times and then dissolved with NaOH (0.5 M) solution for at least 2 h. Extracts were added to scintillation fluid (Scintran Cocktail T; BDH, UK) and counted (Beckman model LS-6000). The difference between the uptake rate of ^{14}C-AIB in the presence or absence of external Na^+ (130 mM) was taken as the Na^+-dependent transport rate. Cell proteins were measured by a modified Lowry method.

2.3. Kinetic and statistical analysis

For a simplified version of the Na^+-coupled transport where Na^+ binds the transporter first, the rate of amino acid transport may assume the following form [16]:

$$v = V_{max} \cdot [Na^+]/([Na^+] + K_m(Na) + k[Na^+]) \quad (1)$$

where v is velocity, $[Na^+]$ is the external Na^+ concentration, $K_m$and V_{max} are the Michaelis constant and maximum velocity for saturable uptake, respectively, and k is the non-saturable parameter. Eq. (1) and other kinetic models were fit to the uptake data using the GraphPad InPlot (Version 4.0) program (GraphPad Software, San Diego, CA, USA). The goodness of the fit was indicated by the square of the correlation coefficient (r^2). Other analysis of the data was performed using an unpaired Student's t-test and significance level was set at $P < 0.05$.

2. Materials

The culture medium MCDB 107 was purchased as powder from J.R. Scientific (Woodland, CA). Fetal bovine serum was obtained from HyClone (Logan, UT). Partially purified porcine-derived growth factors were prepared as described [15] and biological activity determined before use. 2-Amino[1-^{14}C]isobutyric acid (specific activity 55.3 mCi/mmol, batch 72) was purchased from Amersham (Arlington Heights, IL). MeAIB (2-methylaminoisobutyric acid) was from Aldrich Chemical (Milwaukee, WI). All other chemicals were from Sigma (St. Louis, MO).

3. Results

The time-course of ^{14}C-AIB uptake is shown in Fig. 1 in the presence (○) or absence (●) of external Na^+. The uptake was linear up to 20 min, gradually the uptake rate decreased and the accumulation reached plateau at 1.5 h (data not shown). Thus, a 10 min uptake period was selected for the following experiments except Fig. 4. In the absence of external Na^+, the uptake was significantly lower than that with normal (130 mM) Na^+. The Na^+-independent uptake of AIB accounted for 22%. The uptake of AIB in the presence of various external Na^+ concentrations is illustrated in Fig. 2. The rate of uptake was very sensitive to external Na^+ and the line was drawn by fitting the data using Eq. (1) as described in Materials

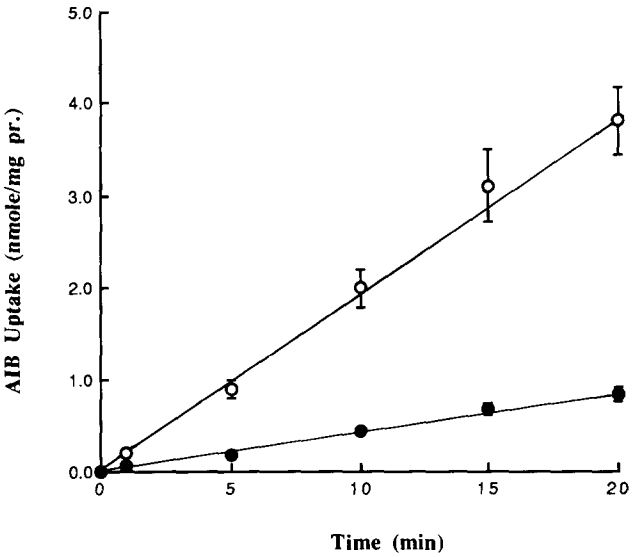

Fig. 1. Time-course of ^{14}C-AIB (18 μM) uptake in the presence (○) and absence (●) of external Na^+. Values of each point were means of three independent determinations each in triplicate, S.E. was shown by a vertical line when greater than the size of the symbol. Linear regression coefficients were better than 0.99 for each line. mg pr, mg cellular protein.

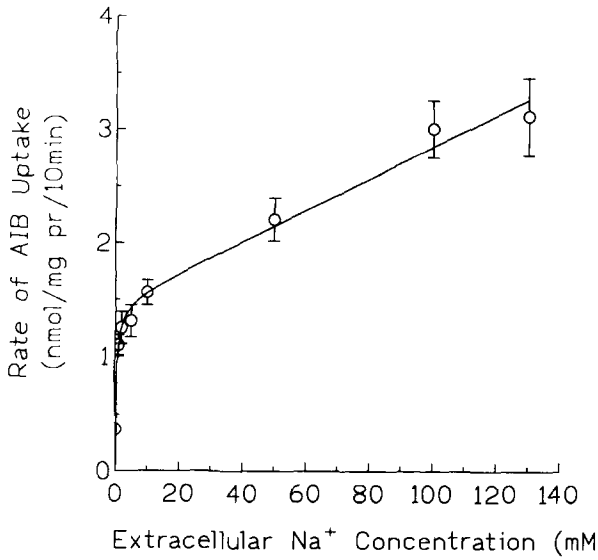

Fig. 2. AIB (18 μM) uptake as a function of extracellular Na^+ concentrations. External Na^+ was replaced with equimolar choline chloride. Values of each point represented the means of four measurements each and S.E. was shown by a vertical line. The curve was drawn from a least-squares fit of Eq. (1).

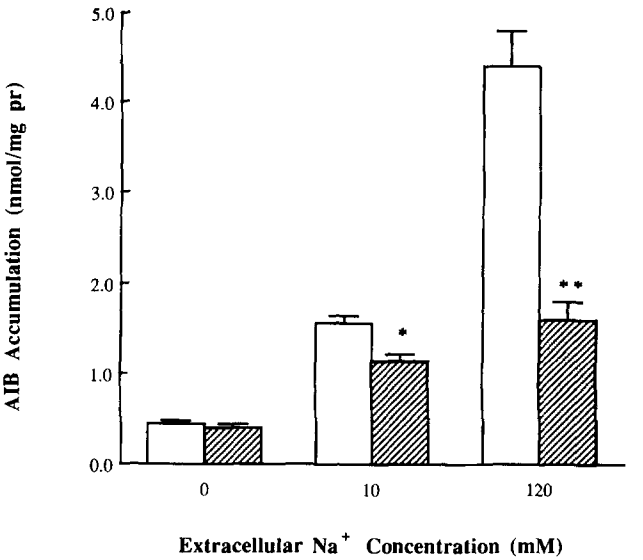

Fig. 4. Effect of ouabain (1 mM) preincubation (1 h) on AIB accumulation at different external Na^+ concentrations. Preincubated without (open column) and with (hatched column) ouabain for 1 h and ^{14}C-AIB was then added to the cells for another hour. A single experiment with six determination each was shown. * $P < 0.005$; ** $P < 0.001$; indicated difference between control (open) and ouabain-treated (hatched).

and methods. It appeared that both saturable and non-saturable components of AIB uptake existed and the external Na^+ concentration required for half-maximal uptake rate of the saturable component (K_m(Na)) was estimated to be 0.40 ± 0.34 mM. The magnitude of the non-saturable component was comparable to V_{max} (0.147 ± 0.0188 nmol/mg protein per min) at 130 mM external Na^+. These data suggest that AIB is mainly transported via Na^+-dependent mechanisms.

Fig. 3 illustrates the kinetics of the AIB transport system of HUVEC. With normal external Na^+ concentration, in the range of low external AIB concentrations, the Lineweaver-Burk plot of the rate of Na^+-dependent AIB transport yielded a straight line with a correlation coefficient of 0.9811 (Fig. 3). Within the range tested, the estimated values for V_{max} and K_m were 0.51 ± 0.12 nmol/mg protein per min and 0.080 ± 0.011 mM, respectively. The Na^+-dependent AIB

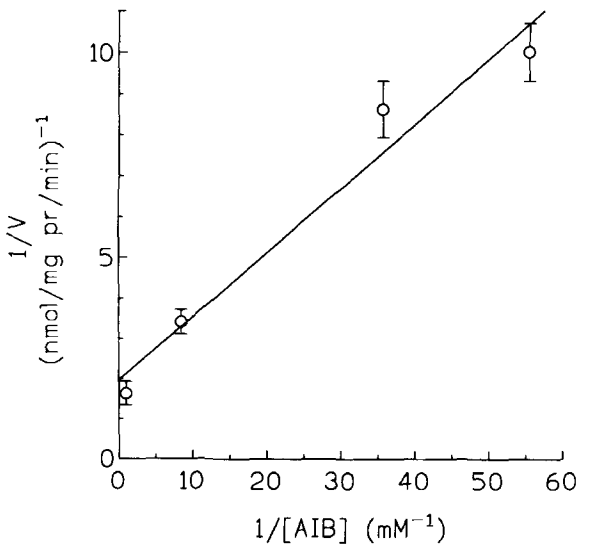

Fig. 3. Kenetics of Na^+-dependent AIB influx. The initial rate of Na^+-dependent AIB uptake fitted by a Lineweaver-Burk plot.

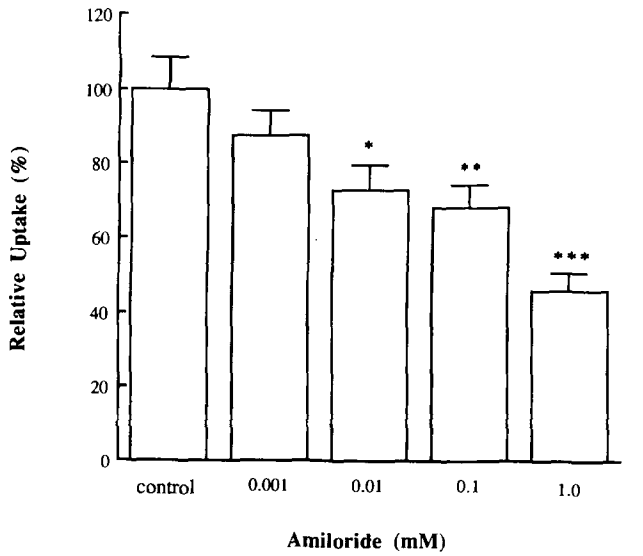

Fig. 5. Effect of amiloride on AIB uptake. Control contained 0.01% of DMSO (vehicle for amiloride) without amiloride and was taken as 100%. * $P < 0.05$; ** $P < 0.025$; *** $P < 0.005$; indicated difference when compared with control. Two determinations of triplicate at each point.

uptake was also inhibited ($P < 0.001$) by 1 mM 2-(methylamino)isobutyric acid (data not shown), a model compound for system A of amino acid transport in many cellular systems including HUVEC [8].

Ouabain, a specific inhibitor of Na^+/K^+ pump, is known to increase cellular Na^+ content of cultured endothelial cells [17]. Preincubation of ouabain for 1 h did not significantly inhibit the initial rate of AIB uptake (10 min pulse, data not shown). However, we found that following the preincubation of 1 mM ouabain, the accumulation of AIB (for 1 h) was significantly inhibited in the presence of external Na^+ (Fig. 4). In the presence of normal external Na^+, a 63% inhibition was reached by ouabain. The small, Na^+-independent accumulation of AIB was not altered. We have also examined the effect of amiloride, a diuretics known to block Na^+ channel in transporting epithelia [18], on the rate of Na^+-dependent AIB uptake in HUVEC. The results are summarized in Fig. 5. Amiloride caused a dose-dependent inhibition of the rate of AIB uptake and reach a 55% inhibition at 1 mM concentration.

4. Discussion

Our results show that HUVEC contain a Na^+-dependent transport system for 2-aminoisobutyric acid (AIB) which is sensitive to agents that directly or indirectly alter the electrochemical gradient of Na^+ accross the cell membrane. Bussolati et al. [8] have shown that in the secondary subcultures of HUVEC, Na^+-dependent MeAIB transport is adaptively enhanced by amino acid starvation. Since the depletion of intracellular amino acid pool is significant when HUVEC is incubated in amino acid-free medium, about half reduced in 90 min as detected by ninhydrin-positive substances [8]; the reported discrepancy [4–6,8] may partly due to the different conditions assumed by the cells for the transport assay. The non-metabolizable alanine analog AIB has been widely used as a model compound for Na^+-dependent, trans-inhibitable system A for the transport of neutral amino acids with small or unbranched sidechains in vitro as well as in vivo studies (for review, see Refs. [13,14]). In the present study, we also employed secondary subcultures (6–10th passage) but the AIB uptake assay was performed shortly (10 min–1 h) after removing from the complete growth media. Thus there should be little, if any, enhancement from 'starvation'. This was supported by the observation that initial rates of AIB uptake were similar whether 10 min or 1 h preincubation was chosen. Under these 'near-basal' conditions, we found that the saturable AIB uptake has a high sensitivity for external Na^+ (Figs. 1 and 2) and that the Na^+-dependent portion of the uptake accounted for about 80% of total AIB uptake. Various kinetic models have been proposed to deduce mechanisms for the Na^+-coupled transport [16,19,20], although our data do not allow detailed analysis for the nature of the coupling, the results (Figs. 2 and 3) are consistent with a saturable Na^+/AIB cotransport system. Furthermore, following ouabain preincubation which may depolarize the membrane potential and/or elevate cellular Na^+, both processes can reduce the electrochemical gradient of Na^+ across the membrane, the accumulation of AIB in HUVEC was significantly reduced in the presence of external Na^+ (Fig. 4). This is consistent with the energetics of Na^+-coupled transport where the electrochemical gradient of Na^+ across the cellular membrane serves as the driving force for the accumulation of Na^+-dependent amino acids [19,21]. These data from the AIB uptake are supportive for the presence of a transport activity characterized by MeAIB uptake as system A in HUVEC [8].

Due to the low abundance of transport proteins in membranes and the overlapping specificities for several amino acids of different transport systems, the molecular nature of the Na^+-dependent amino acid transport system in mammalian cells is not well understood [22]. Our preliminary data have shown that amiloride, an epithelial Na^+ channel blocker [18], could inhibit the activity of AIB transport in HUVEC (Fig. 5). However, effects were observed only at relatively high concentration ($> 10\ \mu M$) and even at 1.0 mM concentration the inhibition reached was only 55%. Thus, it is unlikely that the inhibitory effect was exerted on a conductive Na^+ channel since the half-inhibition constant of amiloride was of submicromolar range in renal and lung membranes [18,23]. Furthermore, the direct effect of this action of amiloride should block Na^+ channel and reduce cellular Na^+ concentration, and thus enhance the electrochemical gradient of Na^+ across the plasma membrane. One would expect a stimulation effect. Whether or not the observed inhibition by amiloride was due to its effect(s) on Na^+-H^+ exchanger (similar concentration requirement as observed) and/or directly on amino acid transporter is not known. At high concentration, cellular effects other than transport inhibition, e.g., inhibition of protein synthesis [24], can not be excluded. Further investigation is necessary to illucidate the nature of the inhibition of AIB transport by amiloride.

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