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Characterization of L-arginine uptake by plasma membrane vesicles isolated from cultured pulmonary artery endothelial cells

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Abstract

We investigated the mechanisms of [^{3}H]-L-arginine transport via System y^+ using plasma membrane vesicles derived from cultured pulmonary artery endothelial cells. [^{3}H]-L-arginine uptake into plasma membrane vesicles was Na-independent, sensitive to trans-stimulation, unaffected by proton-conducting ionophores, and selectively inhibited by cationic amino acids. Kinetic experiments performed over a wide range of substrate concentrations revealed only one population of L-arginine transporters with $K_m = 130\,\mu M$. To elucidate the driving force for L-arginine transport, we measured [^{3}H]-L-arginine uptake by plasma membrane vesicles at different transmembrane ion gradients. Plasma membrane vesicles accumulated [^{3}H]-L-arginine only when a membrane potential was imposed across the vesicles, and the velocity of uptake was linearly related to the magnitude of the created membrane potential. The presence of potassium ions inside the vesicles was not essential for uptake of L-arginine into vesicles, but it was essential for trans-stimulation of L-arginine transport. [^{3}H]-L-arginine accumulated in plasma membrane vesicles can be released by agents that dissipate transmembrane potassium gradients (e.g. saponin, gramicidin, and nigericin). Diazoxide and pinacidil, activators of K^+-channels, had no significant effect on [^{3}H]-L-arginine uptake, whereas tetraethylammonium chloride, 4-aminopyridine, and glibenclamide, inhibitors of K^+-channels, caused decreases in [^{3}H]-L-arginine transport by plasma membrane vesicles. This study demonstrates for the first time a specific role for potassium ions in the mechanism of L-arginine transport, particularly in the phenomenon of trans-stimulation. Published by Elsevier Science B.V.

Keywords: L-arginine transporter; System y^+; Plasma membrane vesicle; Pulmonary artery endothelial cell

1. Introduction

The cationic amino-acid L-arginine is the main source for the synthesis of nitric oxide (NO) in many cell types [1,2]. Vascular endothelial cells synthesize NO via a calcium/calmodulin-dependent constitutive NO synthase (eNOS; ecNOS: Type III NOS) [3]. The K_m of eNOS for L-arginine is $< 10\,\mu M$ [4,5], and several studies have reported values for intracellular L-arginine content in cultured endothelial cells that range from 0.1 to 0.8 mM [6–8]. Therefore, eNOS would be expected to be saturated with L-arginine under physiological conditions. However, a number of in-vitro and in-vivo studies indicate that NO production by vascular endothelial cells under physiological conditions can be increased by exogenous

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L-arginine [9–11]. These studies suggest that extracellular L-arginine and/or its transport into endothelial cells play important, and as yet undefined, roles in NO production by the vascular endothelium.

There are four physiological transport systems (y^+, $b^{o,+}$, $B^{o,+}$, and y^+L) that can mediate L-arginine transport across plasma membranes in different cell types [12]. The majority, i.e. 60–80%, of L-arginine transport by pulmonary artery endothelial cells (PAEC) is mediated by the sodium-independent y^+ transport system [13–16]. At present, three members of a family of cationic amino acid transporters (CAT1, CAT2, and CAT2a) with System y^+ transport activity have been cloned [17]. Although they share a common substrate specificity, the tissue specific expression and the role of these proteins in metabolism differ. In voltage-clamped *Xenopus* oocytes injected with CAT1 cRNA, it was demonstrated that properties of the CAT1-mediated uptake of L-arginine [18,19] were consistent with the transport properties of System y^+, described in endothelial cells [15,16].

An extensive review of System y^+ characteristics is published elsewhere [20]. Two distinctive features of System y^+ are:

1. transport activity is sensitive to changes in extracellular K^+, suggesting that L-arginine influx is influenced by membrane potential [14,19,21,22], and
2. L-arginine uptake via System y^+ is subject to trans-stimulation when substrate concentrations are sufficiently high on the opposite side of the membrane.

However, the detailed mechanisms of these phenomena of L-arginine transport via System y^+ in endothelial cells have not been fully defined. This study describes some properties and characteristics of L-arginine transport by plasma membrane vesicles derived from cultured PAEC. Membrane vesicles isolated from mammalian cells have proved extremely useful for the study of transport processes. Some of their advantages include the ability to use well-defined ion gradients and the lack of metabolism of the transported substance. Thus, the transport process may be studied in isolation from other cellular processes. In the present investigation, we examined the effect of ion gradients in the absence of any exogenous energy source on L-arginine transport by endothelial cell plasma membrane vesicles.

2. Materials and methods

2.1. Tissue culture

Endothelial cells were obtained from the main pulmonary artery of 6- to 7-month-old pigs and were cultured as previously reported [13]. Third- to seventh-passage PAEC in monolayer culture maintained in RPMI-1640 medium plus 4% fetal bovine serum and 100 U/ml penicillin, 100 μg/ml streptomycin, 20 μg/ml gentamicin, and 2 μg/ml fungizone were used for all experiments. Cells were identified and characterized as pure endothelial monolayers as previously described [13].

2.2. Preparation of membrane vesicles

The preparation was based on the procedure described by Teitel [23] and modified by Bhat and Block [24]. This procedure yields a preparation that is free of mitochondria and microsomes and has 15-fold enrichment of Na-K ATPase activity [24,25]. Monolayer cultures were scraped with a large rubber spatula into precooled Hanks' balanced salt solution (HBSS). All subsequent operations were at 4°C. The cell suspension was centrifuged at $800 \times g$ for 15 to 20 min, resuspended in Buffer A (0.25 M sucrose, 0.2 mM $MgSO_4$, 10 mM HEPES-Tris (pH 7.4)), and centrifuged again. The cell pellet was resuspended at a ratio of 1 : 9 :: pellet : Buffer A v/v, and the cells were disrupted by nitrogen cavitation using a precooled minicell disruptor. Nitrogen pressure was 600 to 700 psi. After 15 min under pressure, the suspension was delivered dropwise from the instrument. Then the broken cell suspension was centrifuged at $2000 \times g$ for 5 min to remove nuclei, large aggregates, and broken cells. The postnuclear supernatant was centrifuged for 20 min at $85\,000 \times g$. The resulting pellet was resuspended in Buffer A and layered over a discontinuous (15, 30 and 45%) sucrose gradient. The gradient was centrifuged at $100\,000 \times g$ for 1 h at 4°C. The bands at 15 and 30% sucrose interface were collected, diluted with cold 10 mM HEPES–Tris (pH 7.4), and centrifuged at $85\,000 \times g$ for 20 min. The final pellet of plasma membrane vesicles was suspended in Buffer A at a concentration of 3–5 mg of membrane protein/ml, frozen in liquid nitrogen, and stored at −80°C. Transport activity was stable

for at least one month under these conditions. Purity of the preparation was confirmed by electron microscopy.

2.3. Transport assays

Transport assays were performed essentially as described by Rudnick [26] and modified by Bhat and Block [24,25]. Membranes in Buffer A were rapidly thawed at 37°C and diluted into at least 10 volumes of a loading solution. After incubation for 30 min at 4°C, the suspension was centrifuged at $48\,000 \times g$ for 15 min, and the pellet was resuspended in loading solution to a final concentration of 2–3 mg protein/ml. The composition of the loading solution was 140 mM potassium phosphate (pH 6.8) and 1 mM $MgSO_4$, unless stated otherwise. The resuspended pellet solution (30 μl) was added to 470 μl of external solution containing 140 mM LiCl + 1 mM $MgSO_4$ in 10 mM HEPES–Tris, pH 7.4, and 50 μM [^{3}H]-L-arginine ($\approx$ 2000 dpm/pmol) unless indicated otherwise. Inhibitors and ionophores, when present, were usually added to the external solution prior to the addition of the membrane vesicles. After incubation for specific times at 37°C, reactions were terminated by the addition of 5 ml of ice-cold 0.15 M LiCl (stop solution) followed by filtration through fiberglass Whatman GF/C filters presoaked in 0.5% polyethylenimine. The filters were washed four times with 5 ml of washing solution, dried, and counted using liquid scintillation spectrometry. Zero-time blank values (membrane vesicles added after stop solution) were subtracted from all experimental values.

2.4. Intravesicular volume measurement

Intravesicular volume was determined, using 3-*O*-methyl-[^{14}C]-D-glucose (3-MG), according to the method described by Kletzien et al. [27]. Membrane preparations (50–100 μg of protein) were incubated for 30 min at 37°C with concentrations of [^{14}C]-3-MG ranging from 0.026 to 1.04 mM. [^{14}C]-3-MG uptake was terminated by the addition of an ice-cold isosmotic solution of NaCl followed by filtration and washing the filter four times with 5 ml of the same solution. The radioactivity of the dried filters was counted using liquid scintillation spectrometry. Intravesicular water space was calculated from the slope of the line of the dependence of the equilibrium [^{14}C]-3-MG uptake plotted as a function of substrate concentration. The total intravesicular space determined in this manner was found to be 1.14 ± 0.16 μl/mg protein.

2.5. [^{3}H]-Ouabain binding

[^{3}H]-Ouabain binding was used to test the orientation of vesicles. Ouabain will only bind with high affinity to right-side-out vesicles if they are relatively impermeable to small molecules, and to right-side-out as well as inside-out vesicles if they are leaky or broken, or else after permeabilization of vesicles with saponin. Plasma membrane vesicles loaded with 140 mM potassium phosphate (pH 6.8) and 1 mM $MgSO_4$ (30 μl) were incubated with [^{3}H]-ouabain (80 nM) in the external solution (final volume 0.5 ml) containing 140 mM LiCl and 1 mM $MgSO_4$ (HEPES–Tris buffer, pH 7.4) for 30 min at 37°C. The binding reaction was terminated by addition of the ice-cold external solution followed by filtration through fiberglass Whatman GF/C filters. After washing and drying, the filters were counted using liquid scintillation spectrometry. The nonspecific binding of [^{3}H]-ouabain was determined in the presence of 1 mM unlabeled ouabain and was subtracted from the experimental values. The same experiments were performed on plasma membrane vesicles treated with saponin (50 μg/ml). The analysis of [^{3}H]-ouabain binding indicated that $80 \pm 5\%$ of the vesicles were in a right-side-out orientation.

2.6. Protein determinations

Protein was determined by the method of Lowry et al. [28] using albumin as a standard.

2.7. Statistical analysis

Data are presented as means $\pm$ SE. Student's unpaired *t*-tests were used to determine the significance of differences between means, and $P < 0.05$ was considered significant.

3. Results

3.1. Characterization of the uptake of [^{3}H]-L-arginine into plasma membrane vesicles

The data presented in Fig. 1 illustrate the uptake of [^{3}H]-L-arginine into PAEC-derived plasma membrane vesicles suspended in different extracellular solutions. The maximal velocity of uptake was observed in plasma membrane vesicles loaded with potassium phosphate buffer and diluted 16-fold into NaCl solution containing [^{3}H]-L-arginine. Nearly all of the [^{3}H]-L-arginine was transported in a Na-independent manner, because substitution of Li^+ or choline ions in the external solution for Na^+ ions resulted in only a 6% reduction in [^{3}H]-L-arginine uptake by plasma membrane vesicles (Fig. 1 and Table 1). Substitution of Li^+ or choline for Na^+ in the transport assays yielded the same values for [^{3}H]-L-arginine uptake in plasma membrane vesicles. This is in good agreement with data in intact PAEC [15].

To verify that the Na-independent uptake of L-arginine was being mediated exclusively by System y^+, as opposed to System y^+L, which also transports L-arginine in the absence of sodium [12,29], we measured L-arginine uptake in the presence of 100 μM leucine. This concentration of leucine has been reported to inhibit uptake by System y^+L without affecting uptake by System y^+ [12,17]. As shown in Table 2, 100 μM leucine had no effect on L-arginine uptake by vesicles in the presence or absence of a sodium gradient. In addition, *N*-ethylmaleimide (NEM), a sulfhydryl reactive agent that has been reported to inhibit transport via System y^+ and not via System y^+L [29], caused a 55% reduction in L-arginine transport (Table 2).

The uptake of [^{3}H]-L-arginine by System y^+ reached a maximum by 5 min, and then slowly declined as the initial ion gradients were dissipated. In the absence of transmembrane ion gradients, uptake of [^{3}H]-L-arginine into plasma membrane vesicles was highly diminished (Table 1). If the ion gradients are dissipated by the addition of saponin (50 μg/ml), which renders the membranes highly permeable, intravesicular [^{3}H]-L-arginine rapidly effluxes from the plasma membrane vesicles (Fig. 1). The same effect is induced by the ionophore gramicidin (100 μM), which dissipates both Na^+ and K^+ gradients. The

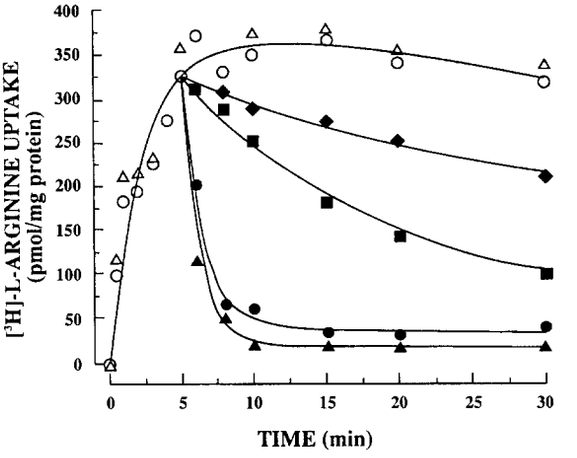

Fig. 1. Transport of [^{3}H]-L-arginine by plasma membrane vesicles. Transport assays were performed as described in Section 2. (△---△) – vesicles (30 μl) loaded with 140 mM potassium phosphate, 1 mM $MgSO_4$, pH 6.8, diluted in 470 μl of 140 mM NaCl containing 1 mM $MgSO_4$ and [^{3}H]-L-arginine; (○---○) – the same only LiCl was substituted for NaCl; (▲---▲) – saponin (50 μg/ml) added to assay mixture at 5 min; (●---●) – gramicidin (100 μM) added to assay mixture at 5 min; (◆---◆) – monensin (5 μM) added to assay mixture at 5 min; and (■---■) – nigericin (5 μM) added to assay mixture at 5 min. Each point represents the mean of at least two experiments performed in triplicate.

ionophore monensin (5 μM), which dissipates primarily the Na^+ gradient, has only a slight effect on [^{3}H]-L-arginine transport, whereas the ionophore nigericin (5 μM), which preferentially disrupts the K^+ gradient, exhibits a much more profound effect (Fig. 1).

The isolated plasma membrane vesicles used in these experiments were osmotically intact as demonstrated by the sensitivity of L-arginine uptake to osmotic pressure (Fig. 2). When the osmolarity of the uptake medium was increased, uptake of [^{3}H]-L-arginine decreased. These results also provide strong evidence that [^{3}H]-L-arginine was accumulating in the intravesicular space rather than simply binding to the membrane surface since the latter would not be expected to vary with the osmolarity of the external solution.

3.2. Kinetics of high-affinity Na-independent arginine transport

Uptake of [^{3}H]-L-arginine by K^+-loaded vesicles, diluted in Li-containing external solution, was found

Table 1
Effects of the composition of the extra- and intravesicular solutions on [^{3}H]-L-arginine uptake by plasma membrane vesicles derived from porcine PAEC [a]

Extravesicular solution [b]	Intravesicular solution [c]	Condition	[^{3}H]-L-arginine uptake (% of control)
140 mM NaCl	140 mM K-phosphate	Membrane potential is created by trans-membrane potassium gradient; Na-gradient is also present (control conditions)	100
140 mM LiCl	140 mM K-phosphate	Membrane potential is created by trans-membrane potassium gradient; Na-gradient is absent	94
100 mM NaCl 40 mM K-phosphate	100 mM LiCl 40 mM K-phosphate	Membrane potential is absent; only Na-gradient is created	7
40 mM K-phosphate 40 mM Na-phosphate 60 mM NaCl	40 mM K-phosphate 40 mM Na-phosphate 60 mM NaCl	Membrane potential and Na-gradient are absent	5
100 mM NaSCN 40 mM NaCl	140 mM LiCl	Membrane potential (interior negative) is created by transmembrane gradient of the highly permeant thiocyanate anion (out > in). Na-gradient is present	72
100 mM NaSCN 40 mM NaCl	140 mM NaCl	Membrane potential is created by trans-membrane gradient of thiocyanate anion. Na-gradient is absent	74

[a] Transport assays were carried out at 37°C with a [^{3}H]-L-arginine concentration of 50 μM as described under Section 2. Reactions were stopped at 3 min by dilution of plasma membrane vesicles into ice-cold external solutions of different compositions. Results in each case are means of at least two experiments performed in triplicate. [^{3}H]-L-arginine uptake under control conditions was 276 ± 25 pmoles/mg protein/3 min.
[b] All extravesicular solutions also contained 1 mM $MgSO_4$ and were buffered with 10 mM HEPES-Tris to pH = 7.4.
[c] All intravesicular solutions also contained 1 mM $MgSO_4$ and had pH = 6.8.

to be saturable at concentrations ranging from 3 to 400 μM (Fig. 3). Analysis of the kinetic data from four separate experiments performed in triplicate, using an Eadie-Hofstee linear transformation, revealed a single high-affinity Na-independent transport system with a K_m of (mean ± standard error) 130 ± 14 μM and a corresponding V_{max} of (mean ± standard error) 694 ± 65 pmol/mg protein/30 s at 37°C. Reported values of K_m for high affinity L-arginine uptake into intact endothelial cells lie in the 6–304 μM range [13,15,16,30].

3.3. Driving force for L-arginine uptake

To elucidate the nature of the driving force for L-arginine uptake, we measured [^{3}H]-L-arginine transport into plasma membrane vesicles in the presence of different ion gradients that were created by varying the composition of the extravesicular and intravesicular solutions (Table 1). [^{3}H]-L-arginine uptake by plasma membrane vesicles was maximal in the presence of a sodium gradient and a membrane potential created by a transmembrane potassium gradient. The sodium gradient was not essential for maximal L-arginine transport because the substitution of Li^+ ions in the external solution for Na^+ ions induced only a small and insignificant reduction in [^{3}H]-L-arginine uptake (Table 1). However, in the absence of a membrane potential, [^{3}H]-L-arginine transport into plasma membrane vesicles did not occur, irrespective of whether a sodium gradient existed (Table 1). A membrane potential (interior negative), created either by a potassium gradient directed outside or a transmembrane gradient of the highly permeant thiocyanate anion directed inside, induced effective [^{3}H]-L-arginine transport into plasma membrane vesicles in the presence as well as in the absence of a sodium gradient (Table 1). The rate of [^{3}H]-L-arginine uptake was related in a linear manner to the value of the membrane potential created by the transmembrane thiocyanate gradient (Fig. 4). [^{3}H]-L-arginine uptakes by plasma membrane vesicles were

Table 2
Effect of L-leucine and NEM on [^{3}H]-L-arginine uptake by plasma membrane vesicles derived from porcine PAEC [a]

Extravesicular solution	Intravesicular solution uptake	[^{3}H]-L-arginine
140 mM NaCl	140 mM K-phosphate	
-leucine		293 ± 8
+ 100 μM leucine		312 ± 8
140 mM LiCl	140 mM K-phosphate	
-leucine		284 ± 22
+ 100 μM leucine		274 ± 20
140 mM NaCl	140 mM K-phosphate	
-NEM		293 ± 8
+ 0.2 mM NEM		131 ± 2 [b]

[a] Transport assays were carried out at 37°C with a [^{3}H]-L-arginine concentration of 50 μM as described in Section 2. Reactions were stopped at 3 min, and results are expressed as pmoles L-arginine/mg protein/3 min. Results are from two experiments performed in triplicate.
[b] $P < 0.01$ vs. NEM.

comparable under approximately the same membrane potential created by potassium or thiocyanate ion gradients.

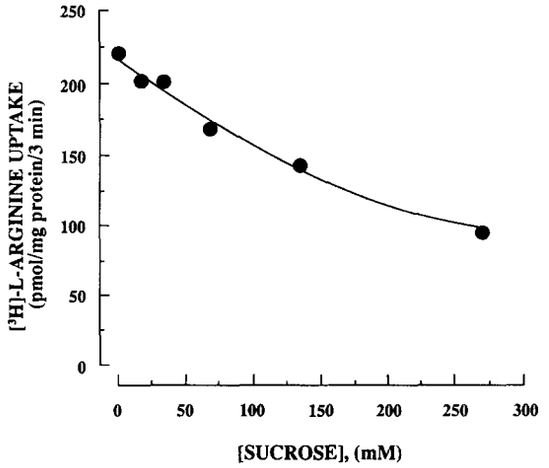

Fig. 2. Effect of the osmolarity of the external solution on [^{3}H]-L-arginine uptake by plasma membrane vesicles. Transport assays were performed as described in Section 2 by diluting vesicles into the uptake medium containing additional concentrations of sucrose to increase osmolarity. The time of incubation with [^{3}H]-L-arginine was 3 min. Each point represents the mean of two experiments performed in triplicate.

3.4. Trans-stimulation of L-arginine influx in plasma membrane vesicles

One of the properties of cationic amino-acid transport mediated by System y^+ is the trans-stimulation of amino-acid uptake when the substrate concentration is sufficiently high on the intracellular (or intravesicular) side of the membrane [12,31]. To study this particular feature of the transport system for L-arginine, we loaded plasma membrane vesicles with potassium phosphate buffer containing various concentrations of L-arginine and then measured uptakes. The uptake of [^{3}H]-L-arginine by vesicles loaded with L-arginine (trans-stimulated uptake) was compared to [^{3}H]-L-arginine uptake by vesicles without intravesicular L-arginine (control uptake) (Fig. 5). A significant trans-stimulatory effect of intravesicular L-arginine emerged at an intravesicular concentration of 0.1 mM and reached a maximal value (increase of ≈ 40%) at an intravesicular concentration of 0.2 mM. Further increases in L-arginine concentration inside the plasma membrane vesicles induced a gradual reduction in the [^{3}H]-L-arginine accumulation in the vesicles, proba-

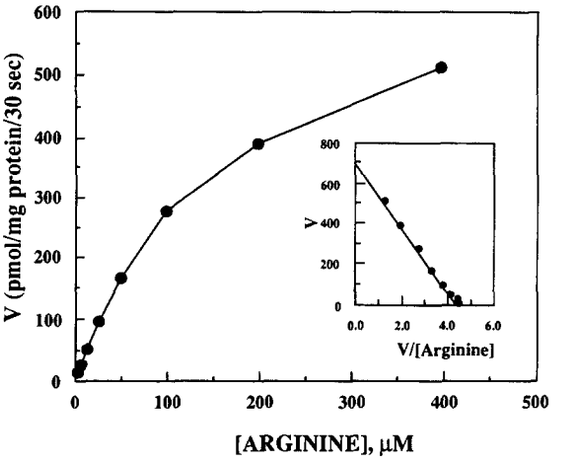

Fig. 3. Kinetics of Na-independent [^{3}H]-L-arginine transport in plasma membrane vesicles isolated from porcine PAEC. Initial rates of [^{3}H]-L-arginine uptake, V, were measured for 30 s at 37°C as described in Section 2 at various concentrations of L-arginine. All measurements were corrected for nonspecific [^{3}H]-L-arginine uptake at each L-arginine concentration (0-time). The data represent mean values of one typical experiment with $K_m = 159$ μM and $V_{max} = 700$ pmol/mg protein/30 s. Inset: accompanying Eadie–Hofstee plot of the data.

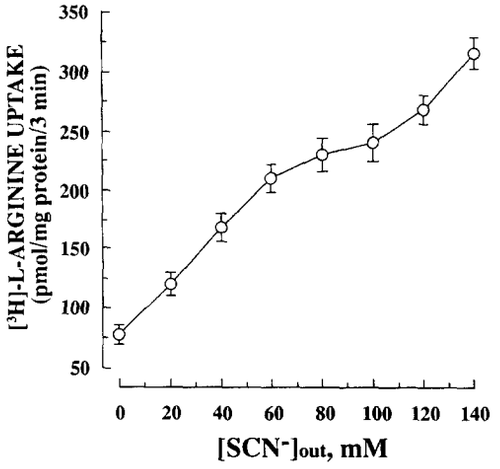

Fig. 4. Dependence of [^{3}H]-L-arginine transport by plasma membrane vesicles on the inward gradient of thiocyanate anions. Membrane vesicles were loaded with 140 mM NaCl + 1 mM $MgSO_4$, in 10 mM HEPES–Tris buffer (pH 7.4) as described in Section 2 and diluted into external solution containing labeled L-arginine and various concentrations of NaSCN. In the solutions with different concentrations of SCN^- ions, NaSCN was substituted for NaCl so that the osmolarities of intra- and extravesicular solutions were the same. The pH of the external solutions was adjusted to 7.4 with 10 mM HEPES–Tris. The incubation time with [^{3}H]-L-arginine was 3 min, and the reaction was stopped by dilution in ice-cold external solution, followed immediately by filtration through GF/C membrane filters as described in Section 2. Each point represents the mean ± S.E.M. of three experiments performed in triplicate.

bly due to leakage of unlabeled L-arginine from the vesicles that competed with extravesicular [^{3}H]-L-arginine for binding sites on the transporter. Nevertheless, [^{3}H]-L-arginine uptake by plasma membrane vesicles loaded with 1 mM unlabeled L-arginine was higher ($\approx$ 20%) than that in control vesicles without L-arginine inside. The effect of trans-stimulation was observed in the presence of an inward-directed sodium membrane gradient (i.e. potassium phosphate buffer inside plasma membrane vesicles and 140 mM NaSCN solution outside) as well as in the absence of this gradient (i.e. potassium phosphate buffer inside and LiCl solution outside). It is noteworthy that we did not observe the trans-stimulatory effect of intravesicular L-arginine on [^{3}H]-L-arginine accumulation in the presence of a membrane potential (created by external 140 mM NaSCN) when a sodium phosphate buffer was substituted for intravesicular potassium phosphate buffer (Fig. 5).

3.5. Modulation of L-arginine transport by amino acids, L-arginine analogs, ionophores, and potassium channel agonists and antagonists

L-Arginine transport was stereospecific and was inhibited markedly by other cationic amino acids (L-lysine, L-ornithine and L-homoarginine) and by the cationic L-arginine analog N^G-monomethyl-L-arginine (L-NMMA), which is a potent inhibitor of eNOS (Table 3). In contrast, neutral amino acids, including 0.1 mM and 1 mM L-leucine, and the neutral L-arginine analog inhibitors of eNOS, NG-nitro-L-arginine (L-NNA) and its methyl ester, L-NAME, were poor inhibitors. Monensin, one of the few ionophores known to selectively complex Na ions [32], induced significantly weaker inhibition of [^{3}H]-L-arginine uptake in plasma membrane vesicles than nigericin, which complexes more readily with K^+ than Na^+. Gramicidin, which dissipates both Na^+ and K^+ gradients, manifested the property of a strong

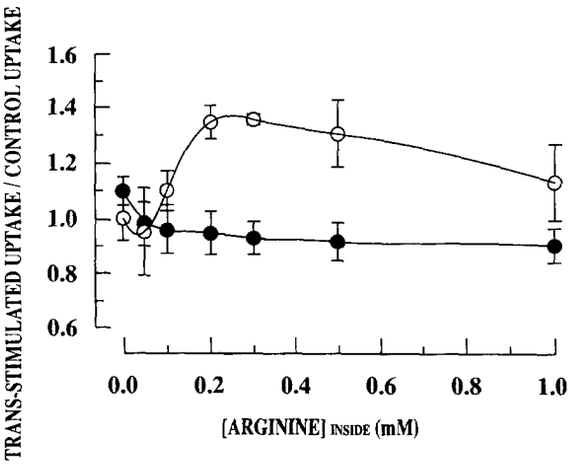

Fig. 5. Trans-stimulation of L-arginine transport by plasma membrane vesicles. [^{3}H]-L-arginine uptake by plasma membrane vesicles loaded with potassium phosphate buffer + 1 mM $MgSO_4$ (pH 6.8) containing different concentrations of L-arginine (○---○). [^{3}H]-L-arginine uptake by plasma membrane vesicles loaded with sodium phosphate buffer plus 1 mM $MgSO_4$ containing different concentration of L-arginine (●- --●). Uptakes were measured in 140 mM NaSCN external solution. The data are expressed as a ratio of [^{3}H]-L-arginine uptake in the presence of a given intravesicular L-arginine concentration (trans-stimulated uptake) to the uptake in control vesicles (no L-arginine inside). Each point represents the mean of at least three experiments performed in triplicate ± S.E.

Table 3
The effects of amino acids, L-arginine analogs, ionophores, and potassium channel agonists and antagonists on [^{3}H]-L-arginine transport by plasma membrane vesicles [a]

Substance	Concentration	[^{3}H]-L-arginine uptake (% of control)
Cationic amino acids		
L-lysine	1 mM	3 ± 2 [d]
L-ornithine	1 mM	5 ± 4 [d]
L-homoarginine	1 mM	5 ± 3 [d]
Neutral amino acids		
L-leucine	0.1 mM	108 ± 7
L-leucine	1 mM	94 ± 5
L-glutamine	1 mM	92 ± 6
L-serine	1 mM	89 ± 8
L-arginine analogs [b]		
L-NMMA	100 μM	14 ± 6 [d]
L-NNA	100 μM	82 ± 10
L-NAME	100 μM	88 ± 11
Cation ionophores		
gramicidin	100 μM	19 ± 5 [d]
monensin	5 μM	78 ± 2 [d]
nigericin	5 μM	25 ± 6 [d]
valinomycin	50 μM	152 ± 17 [d]
H^+-ionophores [c]		
CCCP	50 μM	98 ± 5
FCCP	10 μM	95 ± 3
K^+-channel agonists		
diazoxide	400 μM	89 ± 9
pinacidil	500 μM	87 ± 7
K^+-channel antagonists		
tetraethylammonium chloride	20 μM	74 ± 4 [d]
4-aminopyridine	500 μM	78 ± 3 [d]
glibenclamide	10 μM	72 ± 6 [d]

[a] [^{3}H]-L-arginine transport was assayed at 37°C as described under Section 2. Substances were added to incubation media, and [^{3}H]-L-arginine uptake was initiated by the addition of plasma membrane vesicles. Reactions were stopped at 3 min. Results are means of at least 6 assays in each case. Control uptake of [^{3}H]-L-arginine was 283 ± 22 pmoles/mg protein/3 min (mean ± S.E.M.).
[b] L-NMMA – NG-monomethyl-L-arginine; L-NAME – NG-nitro-L-arginine methyl ester; L-NNA – NG-nitro-L-arginine.
[c] CCCP – carbonyl cyanide m-chlorophenylhydrazone; FCCP – carbonyl cyanide p(trifluoromethoxy)phenyl-hydrazone.
[d] $P < 0.05$ vs. control result.

inhibitor of [^{3}H]-L-arginine transport. On the other hand, the K^+ ionophore valinomycin, which induces hyperpolarization at low external potassium concentrations [33], caused a considerable stimulation of [^{3}H]-L-arginine uptake by plasma membrane vesicles. The proton-conducting ionophores, CCCP and FCCP, had no effect on [^{3}H]-L-arginine transport. Because L-arginine transport is very sensitive to membrane potential [19,33–35], which is largely supported by K^+ gradients, we assessed the effects of both activators and blockers of K^+-channels on [^{3}H]-L-arginine uptake by vesicles. Activators of K^+-channels (diazoxide and pinacidil) had no significant effect on [^{3}H]-L-arginine uptake, whereas blockers of K^+-channels (tetraethylammonium chloride, 4-aminopyridine and glibenclamide) caused decreases in

[^{3}H]-L-arginine transport by plasma membrane vesicles.

4. Discussion

The present investigation was devoted to characterizing the uptake of [^{3}H]-L-arginine by plasma membrane vesicles derived from PAEC. A question that arises in studies such as these is whether the observed interaction between [^{3}H]-L-arginine and the vesicles represents uptake by the vesicles or merely binding to vesicle membranes. Two lines of evidence suggest that, in our experiments, [^{3}H]-L-arginine is taken up inside the vesicles. First, uptake of [^{3}H]-L-arginine decreases when the intravesicular space shrinks upon addition of external sucrose (Fig. 2). This would not be expected to occur if [^{3}H]-L-arginine were merely binding to vesicle membranes. Secondly, the accumulated [^{3}H]-L-arginine effluxed from the plasma membrane vesicles after treatment with the cholesterol-chelating detergent saponin (Fig. 1).

The present study demonstrates that [^{3}H]-L-arginine transport in plasma membrane vesicles is similar in many respects to L-arginine transport in cultured endothelial cells. For example, it is Na^+-independent, sensitive to trans-stimulation, unaffected by proton-conducting ionophores, and selectively inhibited by cationic amino acids. In addition, L-arginine transport is sensitive to *N*-ethylmaleimide and is not affected by micromolar or millimolar concentrations of L-leucine. These transport properties are inherent to System y^+ [12,20,29], which was recently cloned and expressed in *Xenopus* oocytes [18,36]. Our kinetic experiments, performed over a wide range of substrate concentrations, revealed only one population of L-arginine transporters with a $K_m = 130\,\mu M$. We [13] and Green et al. [15] have described an Na^+-dependent transport system for L-arginine in porcine pulmonary endothelial cells that is responsible for 20–40% of total carrier-mediated transport in these cells. However, we did not find any evidence for the existence of this transport system in our plasma membrane vesicles. Although the reason for this is not clear, it may be related to the loss of membrane proteins belonging to this transport system secondary to isolation, purification, and storage of the membrane vesicle preparations.

Our results support the observations of others [14,16,19,21,22] that L-arginine transport by endothelial cells is dependent on membrane potential. First, L-arginine transport into plasma membrane vesicles was not observed in the absence of membrane potential (Table 1). Second, the accumulation of [^{3}H]-L-arginine into plasma membrane vesicles was disrupted by the action of drugs that dissipate transmembrane potassium gradients (e.g. saponin, gramicidin, nigericin) (Fig. 1) and was stimulated by the action of the membrane-hyperpolarizing potassium ionophore valinomycin (Table 3). Because membrane potential is determined in a large part by the transmembrane potassium gradient, the question arises as to whether it is the membrane potential or the chemical concentration gradient for potassium that ultimately drives the uptake of L-arginine. In other words, is it possible that the System y^+ L-arginine transporter works as a K^+/L-arginine exchanger? To test this possibility, we measured [^{3}H]-L-arginine transport into plasma membrane vesicles under conditions where the membrane potential (interior negative) was created not by an outward potassium gradient but by an inward gradient of the highly permeant thiocyanate anion (Table 1, Fig. 4). Our results show that with a membrane potential, [^{3}H]-L-arginine can accumulate in vesicles even in the absence of potassium ions. This clearly demonstrates that the primary driving force for L-arginine transport is the membrane potential. Additional evidence in favor of this conclusion follows from our experiments with potassium channel antagonists, which, by blocking potassium channels, would be expected to depolarize vesicular membranes and reduce [^{3}H]-L-arginine uptake (Table 3). If potassium/L-arginine exchange were driving L-arginine uptake, then the use of potassium channel blockers would have been expected to increase intravesicular concentrations of potassium and, in turn, L-arginine influx, which did not occur.

In addition to contributing to the membrane potential that is the driving force for L-arginine influx, our results indicate that potassium ions play an important role in the phenomenon of trans-stimulation – i.e. the increased uptake of L-arginine in the presence of L-arginine on the opposite (trans) side of the membrane. Existing models of trans-stimulation predict that the conformational change of the carrier occurs much more rapidly in the presence of substrate and,

thus, the return of the transporter to the *cis*-conformation is slow when the substrate concentration on the transmembrane side is low [37]. This, of course, will result in a low level of amino-acid influx (i.e. uptake) from the cis-side. In our experiments, we could not observe a trans-stimulatory effect of intravesicular L-arginine on [^{3}H]-L-arginine uptake in the absence of intravesicular potassium ions (Fig. 5). The precise role that potassium ions play in trans-stimulation is not clear from our studies, but it is possible that the binding of potassium to the L-arginine transport protein increases the affinity of the amino-acid recognition site for L-arginine and promotes the reorientation of the loaded transporter from the trans- to the cis-face of the membrane. Such a mechanism can explain the experimental observation that K^+-depolarization activates the efflux of pre-accumulated [^{3}H]-L-arginine from endothelial cells only in the presence of extracellular substrate (unpublished observations). This property of the L-arginine transporter provides physiological protection against depletion of intracellular L-arginine since concentrations of extracellular potassium that are lower than intracellular potassium will protect against efflux of intracellular L-arginine even during long periods of starvation.

Because L-arginine transport is dependent upon membrane potential, which is supported by K^+ gradients, we tested the effects of both activators and blockers of K^+-channels on [^{3}H]-L-arginine uptake by plasma membrane vesicles. Contrary to our initial expectations, we did not observe increased [^{3}H]-L-arginine uptake by vesicles treated with K^+-channel agonists. However, this surprising result may support the concept that membrane potential is the driving force for L-arginine transport across cellular membranes. There are at least four types of endothelial K^+-channels, two of which are voltage gated (inwardly rectifying K^+-channel and transient (A type) K^+-channel) and two of which are ligand gated (Ca^{2+}-dependent K^+-channel and ATP-sensitive K^+-channel). Under physiological conditions, the resting membrane potential of endothelial cells is determined by the activity of the inwardly rectifying K^+-channels, which are activated by hyperpolarization [38]. A physiologically important role of the inward rectifier K^+ current is to help set the resting membrane potential. Rapid hyperpolarization of endothelial cells induced by such stimulants as bradykinin [39], acetylcholine [40], or substance P [41] is followed by a sustained depolarization due to the activation of the inward rectifier K^+-channels. If the inward rectifier K^+-channels retain their properties in isolated plasma membrane vesicles, the addition of K^+-channel activators will, after a short-term hyperpolarization, induce slight membrane depolarization and, as a result, no significant changes in [^{3}H]-L-arginine uptake by vesicles.

The results of these studies demonstrate that plasma membrane vesicles isolated from cultured lung endothelial cells retain most, if not all, of the functional and pharmacological characteristics of the System y^+ L-arginine transport. In addition, these studies demonstrate for the first time a specific role for potassium ions in the mechanism of L-arginine transport, particularly in the phenomenon of trans-stimulation. We believe that this study represents a first important step toward a more detailed understanding of the molecular and mechanistic aspects of L-arginine transport.

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