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Effect of extracellular potassium on amino acid transport and membrane potential in fetal human fibroblasts

Ovidio Bussolati [a], Philip C. Laris [b], Nicola Longo [a], Valeria Dall'Asta [a], Renata Franchi-Gazzola [a], Guido G. Guidotti [a] and Gian C. Gazzola [a,*]

[a] *Istituto di Patologia Generale, Università di Parma, Via Gramsci 14, 43100 Parma (Italy) and* [b] *Department of Biological Sciences, University of California, Santa Barbara, CA 93106 (U.S.A.)*

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The distribution ratio of the lipophilic cation tetraphenylphosphonium (TPP^+) has been used to estimate the electrical potential difference across the plasma membrane in cultured human fibroblasts. These cells exhibit a membrane potential markedly influenced by the diffusion potential of K^+. High extracellular potassium concentrations depolarize human fibroblasts and depress the activity of transport systems A, ASC (both serving for zwitterionic amino acids), X_{AG}^- (for anionic amino acids), and y^+ (for cationic amino acids). High doses (100 μM) of the K^+-ionophore valinomycin hyperpolarize the cells. This condition enhances the activity of systems A, ASC and y^+. Transport systems L (for neutral amino acids) and x_C^- (for anionic amino acids) are insensitive to changes in extracellular K^+ or to valinomycin. System X_{AG}^- is inhibited by the addition of 100 μM valinomycin, but the effect of the ionophore appears to be potential-independent. These results indicate that: (a) the activity of systems L and x_C^- is potential-independent and (b) the activity of systems A, ASC, X_{AG}^- and y^+ is sensitive to alterations of external $[K^+]$ associated to changes in membrane potential.

Introduction

A number of different amino acid transport systems have been characterized in the human fibroblast with respect to their substrate specificity, sensitivity to inhibitors, distribution at steady state, Na^+ dependence, presence of trans-effects and specific mechanisms of regulation [1–8]. With the exception of system A [9], however, little attention has been given to the possible influence of the electrical potential difference across the cell membrane on the activity of these systems in the fibroblast. This area has probably been neglected because of the difficulty in measuring the membrane potential in these cells.

Since the influence of the membrane potential on amino acid transport is well known in other biological models, we sought to extend the characterization of the amino acid transport systems described in the human fibroblast so as to include dependence on membrane potential. The distribution ratio of the lipophilic cation TPP^+ [10] across the plasma membrane of the human fibroblast has been used to determine the experimental conditions required to change the membrane potential as well as to monitor changes of this parameter. The changes in membrane potential were then compared to results obtained with studies of transport activity performed under the same experi-

* To whom correspondence should be addressed.

Abbreviations: TPP^+, tetraphenylphosphonium; MeAIB, 2-(methylamino)isobutyric acid; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

mental conditions. The transport systems studied here together with their representative substrates included system A (2-(methylamino)isobutyric acid and L-proline), ASC (L-alanine), X^-_{AG} (L-aspartate), y^+ (L-arginine), x^-_C (L-cystine and L-glutamate) and L (L-leucine). This choice provides a broad spectrum of substrates (neutral, anionic, cationic) and of operative mechanisms (Na^+-dependent (A, ASC, X^-_{AG}) vs. Na^+-independent systems (y^+, x^-_C, L); trans-inhibited (A) vs. trans-stimulated systems (y^+, L)). Hence this study may be of very general significance.

Materials and Methods

Cell culture. Fetal human fibroblasts obtained from a 10-week gestational age, karyotypically normal male abortus, were routinely grown in 10-cm diameter dishes in Medium 199 containing 15% fetal calf serum and antibiotics (penicillin 100 I.U./ml; streptomycin 100 μg/ml). The conditions of culturing were: pH 7.4; atmosphere 5% CO_2 in air; temperature, 37°C. For the experiments cells were seeded in 24-well plates (COSTAR) and used when cultures reached 35 $\pm$ 10 μg of protein/cm^2. The culture medium was renewed every 72 h and also 48 h before the experiment. Fetal human fibroblasts were used between the 4th and the 15th passage in vitro.

Experimental techniques. Measurements of solute uptake were performed according to the technique described by Gazzola et al. [11]. Cells were washed twice in modified Earle's balanced salt solution and incubated with the various radioactively labelled solutes for the times and in the conditions described for each experiment. Ionophores were added as stock solutions in ethanol (valinomycin 5 mM; 2,4-dinitrophenol 20 mM). The experiments were stopped with two rapid rinses in 3 ml ice-cold isotonic NaCl and the cells were extracted with 0.5 ml ice-cold ethanol (for the TPP^+ experiments) or with 0.2 ml 10% trichloroacetic acid (for the experiments with other solutes). Extracts were added to 2.5 ml of scintillation fluid and counted for radioactivity with a Packard 460C liquid scintillation spectrometer. Extracted cell monolayers were dissolved with 0.5% sodium deoxycholate in 1 M NaOH and proteins were determined using a modified Lowry procedure [12].

Cell water was estimated by measuring the 3-*O*-methylglucose content (nmol/mg protein) of the cells at intervals corresponding to some of the experimental time courses performed in studies of solute uptake [13]. In order to determine intracellular ionic concentrations, cells were incubated in modified Earle's balanced salt solution in the conditions indicated below, then washed rapidly twice (< 5 s) with ice-cold 0.3 M sucrose (3 ml). Cells were dried overnight at 60°C and extracted with a hypotonic solution of CsCl (10 mM). Ion contents (nmol/mg protein) in the extraction fluid were determined with a Varian AA-275 atomic absorption spectrophotometer using appropriate solutions of NaCl and KCl in hypotonic CsCl (10 mM) as standards. Concentrations were then calculated using appropriate values of cell water.

Materials. Fetal calf serum, growth medium and antibiotics were purchased from Gibco. L-[^{35}S]Cystine hydrochloride (215 Ci/mol); L-[5-^{3}H]proline (30 Ci/mmol); L-[5(n)-^{3}H]arginine hydrochloride (10.8 Ci/mmol); L-[2,3-^{3}H]aspartic acid (15 Ci/mmol); L-[2,3-^{3}H]alanine (35 Ci/mmol); L-[4,5-^{3}H]leucine (61 Ci/mmol) and tetra-[^{3}H]phenylphosphonium bromide (24 Ci/mmol) were from Amersham; L-[1-^{14}C]glutamic acid (55.3 Ci/mol); 3-*O*-methyl-D-[U-^{14}C]glucose (329 Ci/mol) and 2-[1-^{14}C]methylaminoisobutyric acid (53.5 Ci/mol) were obtained from New England Nuclear. Sucrose was purchased from Aristar-BDH and 2-(methylamino)isobutyric acid from Aldrich. The source of all the other chemicals was Sigma.

Results

Cell water and intracellular ion concentrations

The experiments planned in the present study involved the incubation of human fibroblasts at different external concentrations of K^+ in the presence of ionophores. Since there was the possibility that cell water and intracellular ions could change under the experimental conditions employed, these parameters were estimated at different external concentrations of K^+ in the presence and absence of valinomycin and 2,4-dinitrophenol.

Fig. 1 shows that, after a 5 min incubation in external [K^+] ranging between 5 and 150 mM, cell water did not change appreciably. Cell water remained fairly stable at longer times of incubation

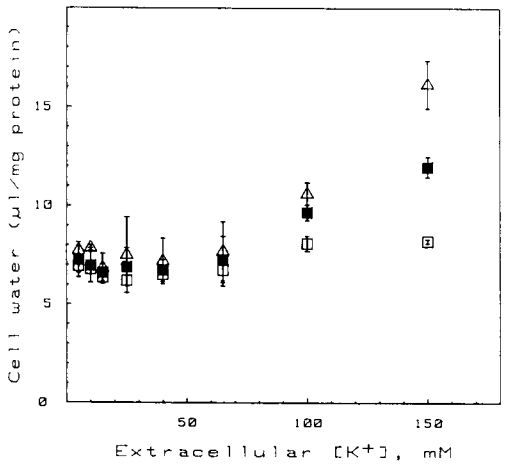

Fig. 1. Cell water in fetal human fibroblasts. Effect of the incubation in media at different K^+ concentrations. Fetal human fibroblasts were incubated for 90 min at 37°C in Earle's balanced salt solution with 15% fetal calf serum, pH 7.4, in an atmosphere of air/CO_2 (95:5). After this period, the incubation medium was substituted with a modified Earle's mixture buffered at pH 7.4 with 20 mM Tris-HCl, in which K^+, at the concentrations indicated, replaced Na^+ ($[Na^+]_o + [K^+]_o = 150$ mM) and 1 mM 3-*O*-methyl-D-[^{14}C]glucose (0.5 µCi/mmol) was added. After 5 min (□), 20 min (■) or 40 min (△) the cells were washed and extracted as described under Materials and Methods. Points are means of three determinations with S.D.

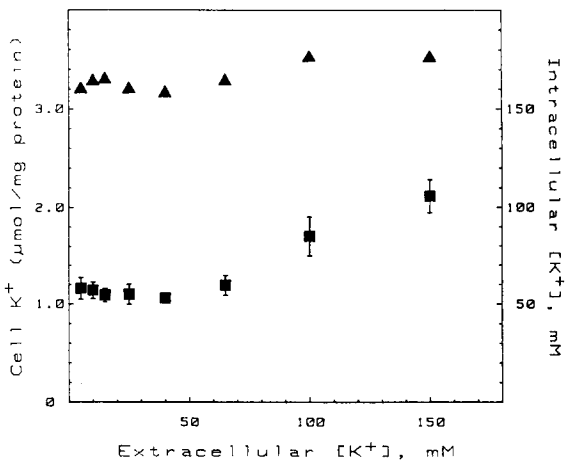

Fig. 2. Intracellular potassium in fetal human fibroblasts. Effect of the incubation in media at different K^+ concentrations. After the preincubation period, the medium was substituted with a modified Earle's mixture (see Fig. 1) in which K^+, at the concentrations indicated, replaced Na^+ ($[Na^+]_o + [K^+]_o = 150$ mM). After 20 min the cells were washed and extracted as described under Materials and Methods. Results, expressed both as µmol/mg of protein (■, with S.D. indicated) and as mM (▲), are means of three determinations.

(20 and 40 min) when $[K^+]$ did not exceed 65 mM, whereas it increased at higher potassium concentrations (100 and 150 mM) [14]. The addition of valinomycin (100 µM) or 2,4-dinitrophenol (500 µM) did not alter this pattern.

A study of the intracellular K^+ concentration as a function of extracellular $[K^+]$ was made at various incubation times in the absence or in the presence of valinomycin. Cells incubated for 20 min at external $[K^+]$ ranging between 5 and 150 mM changed their K^+ content from 1.16 to 2.11 µmol/mg protein (Fig. 2). This corresponded, in terms of intracellular K^+ concentrations, to very little change; internal $[K^+]$ remained quite constant (around 160 mM) up to 65 mM external $[K^+]$ and increased slowly thereafter reaching values of 176 mM at 150 mM external $[K^+]$ (Fig. 2). The increase in cell water observed after 20 min incubation in high external $[K^+]$ (Fig. 1) paralleled the increase in µmol K^+/mg protein and prevented large changes in K^+ concentration. Intracellular Na^+ concentration, on the other hand, fell from 22 mM to 4 mM upon a 20 min incubation of the cells in a medium containing 150 mM K^+. The addition of valinomycin (100 µM) did not affect the intracellular concentrations of K^+ and Na^+ under these experimental conditions.

Measurement of TPP^+ distribution ratios

The time-courses of uptake of increasing TPP^+ concentrations are shown in Fig. 3. The TPP^+ distribution ratios at each incubation time and concentration of the lipophilic cation were markedly higher when the cells were incubated in low-K^+ (5 mM, panel A) than in high-K^+ (150 mM, panel C) medium. With both K^+ concentrations, the TPP^+ ratio was higher (and steady state was reached later) when low concentrations of the lipophilic cation were used. In the presence of 2,4-dinitrophenol (500 µM) the effect of the extracellular K^+ concentration on TPP^+ distribution ratios was maintained (Fig. 2, panels B and D) and the time required to reach steady state was shortened. 2,4-Dinitrophenol markedly reduced the TPP^+ distribution ratio either in low-K^+ or in

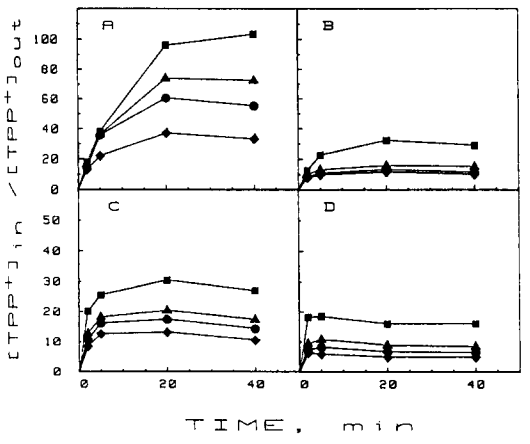

Fig. 3. Time-course of the uptake of different concentrations of TPP^+ in fetal human fibroblasts. After the preincubation period, the cells were incubated in a modified Earle's mixture (see Fig. 1) at 5 mM (panels A and B) or at 150 mM (panels C and D) extracellular $[K^+]$ containing various concentrations of $[^3H]TPP^+$ (■, 0.04 μM, 2.5 Ci/mmol; ▲, 0.2 μM, 0.5 Ci/mmol; ●, 1 μM, 0.1 Ci/mmol; ◆, 5 μM, 0.02 Ci/mmol) in the absence (panels A and C) or in the presence (panels B and D) of 500 μM 2,4-dinitrophenol. During the uptake of TPP^+ ethanol was 2.5%. At the indicated times, the cells were washed and extracted as described under Materials and Methods. Points are means of three determinations.

high-K^+ medium at all external TPP^+ concentrations used. This result could be explained through the dissipation of the electrical potential difference across the inner mitochondrial membrane caused by the ionophore.

Fig. 3 also shows that, when the cells were incubated in a high-K^+ (150 mM) medium in the presence of 2,4-dinitrophenol, the TPP^+ cell/medium distribution ratio was greater than 1 at all the external TPP^+ concentrations used. If the observed TPP^+ distribution were contributed only by the plasma membrane potential, depolarized cells (as those incubated in 150 mM external K^+ whose membrane potential is probably close to 0) should exhibit TPP^+ distribution ratio approaching 1. Therefore, the data shown in panel D suggest that a large fraction of TPP^+ uptake, not related either to the plasma membrane potential or to the potential across the mitochondrial membrane, is presumably due to non-specific binding. The results shown in panel D also indicate that the TPP^+ distribution ratio attributable to non-specific binding of the lipophilic cation increases with decreasing external TPP^+ concentration.

Fig. 4 shows that the TPP^+ distribution ratio remained nearly constant when external $[K^+]$ was varied between 5 and 15 mM. The ratio decreased steadily when $[K^+]$ was varied from 15 mM to 150 mM. The substitution of choline for Na^+ in the medium had little effect, if any, on the TPP^+ distribution ratio. As expected (Fig. 3), the addition of the H^+-ionophore 2,4-dinitrophenol reduced the TPP^+ distribution ratio without altering the shape of the curve relating TPP^+ distribution ratio to external $[K^+]$.

The K^+-ionophore valinomycin also decreased the TPP^+ distribution ratio in fetal human fibroblasts (Fig. 5). At 5 mM external $[K^+]$, the distribution ratio decreased to a minimum at the lowest concentrations of valinomycin used (2 and 10 μM) and then increased with higher concentrations (50 and 100 μM) of the ionophore. Valinomycin, as 2,4-dinitrophenol, is known to abolish the electrical potential across the mitochondrial membrane. While it is likely that the effect of the lowest concentrations of the K^+ ionophore used depends upon the failure of the mitochondria to concentrate TPP^+, the relative increase of TPP^+ distribution ratio observed at higher valinomycin concentrations can be attributed to a hyperpolari-

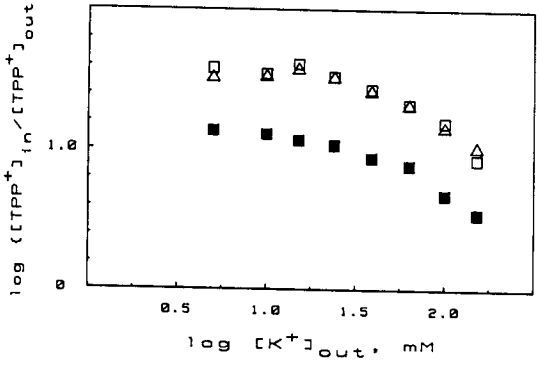

Fig. 4. Dependence of TPP^+ distribution ratio on the extracellular K^+ concentration in fetal human fibroblasts. After the preincubation period, the medium was substituted with a modified Earle's mixture (see Fig. 1) containing 5 μM $[^3H]TPP^+$ (20 Ci/mol) in the absence (open symbols) or in the presence (closed symbols) of 100 μM 2,4-dinitrophenol. For the squares: $[Na^+]_o + [K^+]_o = 150$ mM; for the triangles: $[choline]_o + [K^+]_o = 150$ mM. During the uptake of TPP^+ ethanol was 0.5% (v/v). After 20 min the cells were washed and extracted as described under Materials and Methods. Points are means of three determinations.

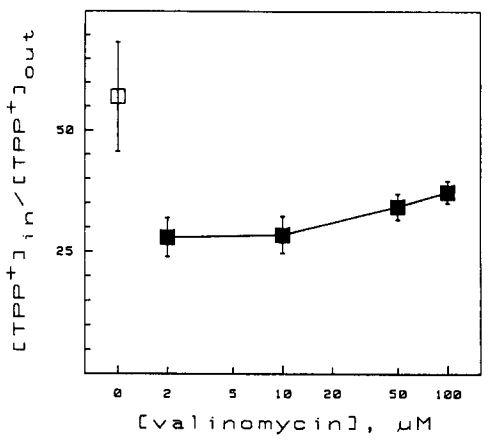

Fig. 5. Effect of valinomycin on the distribution ratio of TPP^+. After the preincubation period, the medium was substituted with a modified Earle's mixture at low (5 mM) extracellular potassium concentration (see Fig. 1) containing 0.2 μM [^{3}H]TPP^+ (0.5 Ci/mmol) in the absence (□) or in the presence (■) of increasing concentrations of valinomycin. During the uptake of TPP^+ ethanol was 2% (v/v). After 20 min the cells were washed and extracted as described under Materials and Methods. Points are means of three determinations.

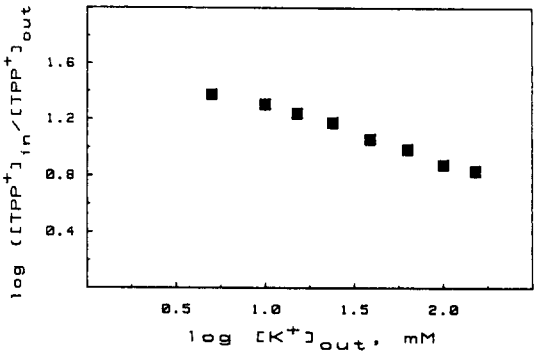

Fig. 6. Dependence of TPP^+ distribution ratio on the extracellular K^+ concentration. Effect of valinomycin. After the preincubation period, fetal human fibroblasts were incubated in a modified Earle's mixture (see Fig. 1) containing 0.2 μM [^{3}H]TPP^+ (0.5 Ci/mmol), 100 μM valinomycin and 5% (v/v) ethanol. After 5 min the cells were washed and extracted as described under Materials and Methods. Points are means of three determinations.

zation of the cell membrane. In support of this interpretation, when the TPP^+ distribution ratio was studied as a function of external [K^+] in the presence of high (100 μM) concentrations of valinomycin (Fig. 6), the relationship between log distribution ratio of TPP^+ and log external [K^+] was nearly linear in the whole range of potassium concentrations tested (5 to 150 mM).

Initial rates of amino acid entry vs. external potassium concentration

The effect of changing external [K^+] on the initial rates of entry of representative substrates for six amino acid transport systems was studied in cultured human fibroblasts (Tables I and II). The influx measurements were performed in a medium containing a constant concentration of Na^+ (50 mM), while K^+ was varied in exchange for choline from 5 to 100 mM. The cells were depleted of the internal amino acid pool before the uptake assay to minimize trans-effects [1]. The transport activity of three Na^+-dependent systems, A, ASC and X^-_{AG}, as well as that of the Na^+-independent system y^+ decreased as the external [K^+] was raised. In contrast, the activity of the

TABLE I

EFFECT OF EXTRACELLULAR POTASSIUM CONCENTRATION ON THE ACTIVITY OF SODIUM-DEPENDENT AMINO ACID TRANSPORT SYSTEMS

Fetal human fibroblasts were depleted of intracellular amino acids (90-min incubation at 37°C in Earle's balanced salt solution additioned with 15% dialyzed fetal calf serum). After this period, initial velocity of entry was measured incubating the cells for appropriate times (60 s for L-proline, 2-(methylamino)isobutyric acid (MeAIB) and L-aspartate; 10 s for L-alanine) in a modified Earle's mixture containing the labelled amino acids. Extracellular concentration of sodium was kept constant at 50 mM while extracellular potassium varied as indicated ($[K^+]_{out}$ + [choline]$_{out}$ = 100 mM). Concentration and specific activity of the substrates were: for L-proline, 0.1 mM, 10 Ci/mol; for MeAIB, 0.1 mM, 5 Ci/mol; for L-alanine, 0.05 mM, 20 Ci/mol; for L-aspartate, 0.01 mM, 200 Ci/mol. For a correct estimation of system ASC transport activity, L-alanine uptake was measured in the presence of 0.5 mM MeAIB. Results shown are menas of three determinations with S.D. Difference between uptakes measured at 5 and 100 mM extracellular [K^+] was significant ($P < 0.01$) for the four amino acid tested (as assessed with analysis of variance).

$[K^+]_{out}$ (mM)	Initial velocity of entry (nmol/ml cell water per min)			
	L-Pro	MeAIB	L-Ala	L-Asp
5	118±5	147±11	792±55	13 ±1.4
24	101±4	119± 2	721±32	9.3±0.1
52.5	76±7	98± 5	570±42	5.6±0.6
100	68±4	76± 4	537±35	3.7±0.6

TABLE II

EFFECT OF EXTRACELLULAR POTASSIUM CONCENTRATION ON THE ACTIVITY OF SODIUM-INDEPENDENT AMINO ACID TRANSPORT SYSTEMS

Depletion and uptake assay were performed as described in Table I. Concentration and specific activity of the substrates were: for L-arginine, 0.02 mM, 100 Ci/mol; for L-cystine, 0.07 mM, 43 Ci/mol; for L-glutamate, 0.05 mM, 40 Ci/mol; for L-leucine, 0.01 mM, 100 Ci/mol. Uptake times were 60 s for L-cystine and L-glutamate, 20 s for L-arginine, 10 s for L-leucine. The difference between uptakes measured at 5 and 100 mM extracellular $[K^+]$ was significant ($P < 0.01$) only for L-arginine (as assessed with analysis of variance).

$[K^+]_{out}$ (mM)	Initial velocity of entry (nmol/ml cell water per min)			
	L-Arg	L-Cystine	L-Glu	L-Leu
5	108 ± 4	35 ± 6	17 ± 0.7	736 ± 47
24	95 ± 4	35 ± 2	18 ± 1	713 ± 54
52.5	84 ± 9	38 ± 5	18 ± 3.4	831 ± 39
100	64 ± 10	40 ± 4	17 ± 1.7	731 ± 79

Na^+-independent systems L and x_C^- remained substantially unaffected upon changes in extracellular K^+ concentration.

Some studies were performed to ascertain whether trans-effects associated with the operation of the various transport systems interfere with the above-mentioned effects. Amino acid transport measurements were therefore performed at low K^+ (5 mM) or at high K^+ (100 mM) before and after exhaustive cell depletion of the internal amino acid pool. Table III shows that the activities of systems A, ASC, X_{AG}^-, x_C^- and L were comparably sensitive to external $[K^+]$ changes either in fed or depleted cells. In contrast, the operation of system y^+ was less affected by changes in external $[K^+]$ in fed (trans-stimulated) than in depleted cells (when trans-stimulation is likely to be minimal). Trans-stimulation increased the uptake of L-arginine by system y^+ more markedly at 100 mM K^+ than at 5 mM K^+, and thus the difference in transport rate recorded in depleted cells at these external $[K^+]$ is diminished in fed cells.

Effects of valinomycin on amino acid transport

Valinomycin has been shown to accelerate the inward transport of site A-reactive amino acids in cultured human fibroblasts [9]. This result has been confirmed in our biological preparation (cultured fetal human fibroblasts) using 2-(methylamino)isobutyric acid as representative amino acid substrate of transport system A [1]. A stimulatory effect was detected in cells incubated in a low-K^+ (5 mM) medium with 100 μM valinomycin (Table IV), but not with lower concentrations (10 μM) of the K^+ ionophore (cf. Table V). Table IV shows also that 100 μM valinomycin enhanced the uptake of L-alanine through system ASC and of L-arginine (system y^+). The entry of L-leucine (site L-reactive substrate) and L-cystine (site x_C^--reactive substrate) were unaffected in the presence of the K^+ ionophore. Valinomycin markedly de-

TABLE III

POTASSIUM DEPENDENCE OF AMINO ACID TRANSPORT IN FETAL HUMAN FIBROBLASTS

Uptake assays were performed in fibroblasts immediately after removal of culture medium 199 (fed cells) or after a 6-h incubation in Earle's balanced salt solution supplemented with 15% dialyzed fetal calf serum (depleted cells). For proline transport assay in depleted cells, 5 μg/ml cycloheximide were added to prevent adaptive enhancement of system A activity [7]. Means of three determinations are shown with S.D. For other details see the legends to Fig. 1 and to Tables I and II. Significance was tested with analysis of variance (n.s., not significant).

Amino acid	System	Initial velocity of entry (nmol/ml cell water per min)					
		Fed cells			Depleted cells		
		$[K^+]_{out} = 5$ mM	$[K^+]_{out} = 100$ mM	P	$[K^+]_{out} = 5$ mM	$[K^+]_{out} = 100$ mM	P
L-Proline	A	73 ± 2	55 ± 1	< 0.01	106 ± 2	79 ± 3	< 0.05
L-Alanine	ASC	1033 ± 40	754 ± 8	< 0.01	916 ± 18	712 ± 67	< 0.05
L-Aspartate	X_{AG}^-	16 ± 1	7 ± 0.5	< 0.01	13 ± 1	6 ± 0.9	< 0.01
L-Arginine	y^+	139 ± 1	124 ± 2	< 0.05	79 ± 2	43 ± 1	< 0.01
L-Cystine	x_C^-	61 ± 5	55 ± 1	n.s.	44 ± 3	45 ± 3	n.s.
L-Leucine	L	2028 ± 445	2081 ± 546	n.s.	735 ± 68	666 ± 62	n.s.

TABLE IV

EFFECT OF VALINOMYCIN (100 μM) ON THE TRANSPORT OF AMINO ACIDS IN FETAL HUMAN FIBROBLASTS

Uptake assays were performed at $[K^+]_{out}$ = 5 mM in the presence or in the absence of valinomycin (100 μM). Ethanol concentration was 2% (v/v) both in controls and in valinomycin-treated cells. Means of three determinations are shown with S.D. For other details, see the legends to Tables I and II. MeAIB, 2-(methylamino)isobutyric acid. n.s., not significant.

Amino acid	System	Initial velocity of entry (nmol/ml cell water per min)		
		Control cells	Valinomycin-treated cells	*P*
MeAIB	A	100 ± 4	126 ± 11	< 0.05
L-Alanine	ASC	771 ± 45	942 ± 103	< 0.05
L-Aspartate	X^-_{AG}	11 ± 0.4	6.6 ± 0.8	< 0.01
L-Arginine	y^+	91 ± 3	108 ± 8	< 0.05
L-Cystine	x^-_C	42 ± 2	41 ± 3	n.s.
L-Leucine	L	781 ± 59	810 ± 33	n.s.

TABLE V

EFFECT OF VALINOMYCIN (10 μM) ON THE TRANSPORT OF 2-(METHYLAMINO)ISOBUTYRIC ACID AND L-ASPARTATE IN FETAL HUMAN FIBROBLASTS

Uptake assays were perforemd at the indicated $[K^+]_{out}$ in the presence or in the absence of valinomycin (10 μM). Ethanol concentration was 0.2% (v/v) both in control and in valinomycin-treated cells. Means of three determinations are shown with S.D. For other details, see the legends to Tables I and II. MeAIB, 2-(methylamino)isobutyric acid. n.s., not significant.

Amino acid	System	Initial velocity of entry (nmol/ml cell water per min)					
		$[K^+]_{out}$ = 5 mM			$[K^+]_{out}$ = 100 mM		
		Control	Valinomycin	*P*	Control	Valinomycin	*P*
MeAIB	A	112 ± 5	116 ± 5	n.s.	65 ± 3	60 ± 2	n.s.
L-Aspartate	X^-_{AG}	19 ± 1	10 ± 1	< 0.01	4.5 ± 0.2	1.9 ± 0.2	< 0.01

pressed the uptake of L-aspartate, a site X^-_{AG}-reactive substrate. This inhibitory effect of the K^+ ionophore was detectable even when its concentration was lowered to 10 μM and persisted in cells incubated in high-K^+ medium (Table V). Valinomycin at 10 μM concentration did not affect 2-(methylamino)isobutyric acid uptake by cells incubated either in a low-K^+ or in a high-K^+ medium.

Discussion

Lipid soluble cations [15] have been used in many studies with different types of tissues, cells, organelles and membrane preparations to evaluate the electrical potential difference across the biological membranes. Although the use of cations as TPP^+ has enabled investigators to monitor qualitative changes in the electrical potential difference across plasma membranes, a number of problems have been recognized when attempts were made to obtain quantitative conclusions from these studies [16–23]. One cannot assume that TPP^+ is simply distributed across the membrane in accordance with the Nernst equation because other factors may influence its cellular uptake. In eukaryotic cells, for example, mitochondrial uptake of TPP^+ can be significant since the electrical potential difference across the inner membrane of this organelle (nearly 200 mV) would be able to maintain a 1000-fold concentration gradient. In addition, the cation may become dissolved in or adsorbed to cellular components. These difficulties can be overcome by: (a) the estimation of mitochondrial uptake of TPP^+ [18–21] or its suppression [22,23] through the use of uncoupler agents such as 2,4-dinitrophenol or CCCP and (b) the subtraction of the non-specific TPP^+ binding from TPP^+ uptake. TPP^+ binding can be estimated as apparent TPP^+ uptake in cells whose

membrane potential approaches zero (i.e., cells incubated in a high-K^+ medium) [16,23]. The combined use of these corrections [23] should allow reliable measurements of cell membrane potential by the Nernst equation provided that a full suppression of TPP^+ mitochondrial uptake is achieved and that the non-specific TPP^+ binding remains constant at different external [K^+].

In our study with human fibroblasts, TPP^+ mitochondrial uptake has been abolished by adding 500 μM 2,4-dinitrophenol (Fig. 3) or valinomycin in excess of 2 μM (Figs. 5 and 6). The non-specific TPP^+ binding has been measured by incubating the cells in a 150 mM K^+ medium in the presence of an agent (2,4-dinitrophenol or valinomycin) capable of dissipating the mitochondrial membrane potential. Under these conditions, the plasma membrane potential is assumed to be equal to E_K, i.e. $61 \times \log(150/176)$ (where these values are the external and the internal K^+ concentrations, respectivley, cf. Results). The TPP^+ distribution ratio equivalent to this residual membrane potential (−4 mV) is 1.2 and any excess must be considered non-specific TPP^+ binding. The non-specific binding of TPP^+ is likely to remain constant at low K^+ (5 mM) and at high K^+ (150 mM) when the external TPP^+ concentration varies between 0.2 and 5 μM; within this range, but not at lower TPP^+ concentrations, the correction of the data shown in panel B of Fig. 3 for the non-specific TPP^+ binding (corrected by subtracting the corresponding values in excess of 1.2 in panel D of the same figure) yields comparable values of TPP^+ distribution ratio (7.2 to 7.5). Therefore, in cultured human fibroblasts, an appropriate correction for non-specific TPP^+ binding requires measurements of TPP^+ uptake performed at external TPP^+ concentrations not lower than 0.2 μM (and, possibly, not exceeding 5 μM).

The same correction procedure can be applied to the data shown in Fig. 6 where the TPP^+ distribution ratio is plotted versus log external [K^+] in the presence of 100 μM valinomycin. When these corrected TPP^+ distribution ratios are plotted against K^+ distribution ratios obtained in the same experimental conditions (Fig. 7), the relationship is compatible with a Nernst-type dependence of log TPP^+ distribution ratio on membrane potential. The slope of the best-fit line (0.856; $r = 0.973$ with $P < 0.01$) is not significantly different from the theoretically expected value of one. If the value of TPP^+ distribution ratio obtained at the lowest external [K^+] is excluded from the fit, the correspondence between the experimental (slope 0.973; $r = 0.984$ with $P < 0.01$) and the expected Nernst-type relationship becomes striking and suggests that the membrane potential deviates somewhat from E_K at low, physiologic extracellular [K^+], even in the presence of very high valinomycin. A similar behavior is typical for many excitable tissues when K^+ permeability is high but not so high that the contribution of other ions to the membrane potential can be disregarded. Fig. 7 indicates that conditions can be defined in which the Nernst equation holds for TPP^+ in cultured human fibroblasts, allowing the calculation of reliable values of membrane potential. When membrane potential is calculated from the corrected TPP^+ distribution ratios derived from the data in Fig. 3 (7.2–7.5, see above), values of −56 or −58 mV are obtained. (Note that these values include −4 mV depending on the assumed residual membrane potential at 150 mM external [K^+]). These values are very different from those reported by other authors [24] for similar cells (cultured human fibroblasts). These authors, however, did not correct their TPP^+ dis-

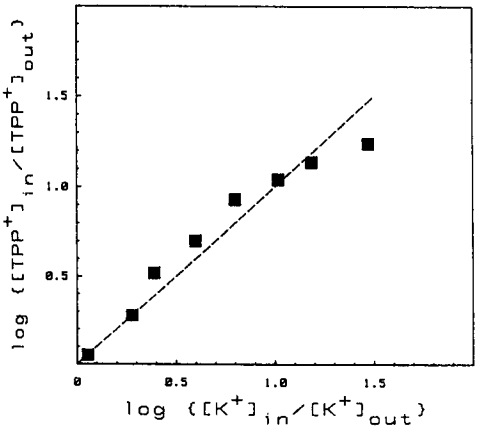

Fig. 7. Relationship between the distribution ratios of TPP^+ and K^+ in fetal human fibroblasts. Values of TPP^+ distribution ratio are obtained from those reported in Fig. 6 after subtracting the non-specific binding of the lipophilic cation from $[TPP^+]_{in}$ (see text). The line represents the theoretical Nernst relationship (slope 1).

tribution ratios for TPP^+ mitochondrial uptake (as recognized by the same authors in a footnote).

The present studies with TPP^+ indicate that fetal human fibroblasts have a membrane potential comparable to that recorded in cells of excitable tissues and suggest that, as in these tissues, the membrane potential is markedly influenced by the diffusion potential of K^+.

The activity of four (A, ASC, X_{AG}^- and y^+) of the six amino acid transport systems previously described in human fibroblasts [1–8] was lowered by increasing the extracellular K^+ concentrations (Tables I and II), i.e. in conditions associated with a decrease of the TPP^+ distribution ratio (Figs. 4 and 6), and hence a decrease of the membrane potential. These observations suggest, but do not prove, that the membrane potential influences the activity of these transport systems for a direct inhibitory effect of K^+ cannot be excluded in these experiments. With a high concentration (100 μM) of valinomycin, however, the initial rate of entrance of amino acids representative of systems A, ASC and y^+ (System X_{AG}^- represents a separate use discussed below) was increased in a low-K^+ medium. Since TPP^+ uptake was also increased under these conditions, the increased rate of amino acids can be correlated with a hyperpolarization. Furthermore a significant linear relationship (Fig. 8) is obtained when v (initial velocity of entry) is plotted against $\exp(-FV/RT)$ for all the substrates of the K^+-sensitive transport systems as would be expected if the initial velocity were dependent on membrane potential [25].

The common feature of these four systems is that their activity is likely to imply or to be associated with the transfer of one or more positive charges into the cell. For Systems A and ASC, which transport zwitterionic amino acids, the stoichiometry of Na^+-substrte cotransport is 1 : 1. For system X_{AG}^-, which carries anionic substrates, this stoichiometry is probably equal to or higher than 2 : 1 [26]. The Na^+-independent system y^+ carries positively charged (cationic) amino acids [5].

The transport of neutral amino acids through system A has been found to vary with membrane potential in several types of cells [27,28], including human fibroblasts [9]. The data presented in Tables I and IV confirm that the activity of system A decreased in fetal human fibroblasts upon cell depolarization caused by an increase of extracellular K^+ and increased upon cell hyperpolarization caused by the addition of high concentrations of valinomycin at low external $[K^+]$. Table III adds that membrane potential did not alter trans-inhibition of system A by internal amino acids.

The activity of system ASC, a Na^+-dependent mediation that also serves for neutral amino acids [1,6], exhibited a similar response to changes of membrane potential, as provoked by varying external $[K^+]$ (Table I) or by adding valinomycin at high concentration (Table IV).

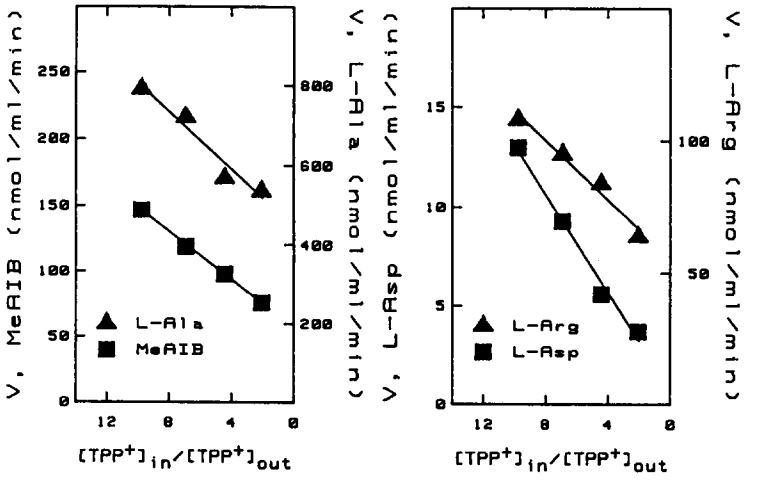

Fig. 8. Relationship between the activity of the K^+-sensitive amino acid transport systems and the TPP^+ distribution ratio. Values of amino acid transport activity, taken from Tables I and II, are plotted against TPP^+ distribution ratio, as a measure of (electrochemical activity coefficient), defined as $\exp(-FV/RT)$. Values of TPP^+ distribution ratio are obtained from the data reported in Fig. 4 (uptake of TPP^+ in the presence of 100 μM 2,4-dinitrophenol) after subtraction of the non-specific binding of the lipophilic cation from $[TPP^+]_{in}$. MeAIB, 2-(methylamino)isobutyric acid.

The influence of $[K^+]$ and of membrane potential on the Na^+-dependent transport of anionic amino acids has been studied in vesicles derived from brain [29] and from the brush-border membrane of kidney cortex cells [30,31], as well as in red blood cells [32]. Those preparations contain a transport system which closely resembles system x^-_{AG} [4,7] in stereoselectivity anomalies, dependence upon sodium and kinetic behavior. A sensitivity to membrane potential has been detected in all these biologic preparations except rabbit kidney brush-border vesicles [31], where the transport of anionic amino acids was potential independent but still sensitive to a K^+ transmembrane gradient. In our cells, the activity of system X^-_{AG} was influenced by external $[K^+]$ (Table I) decreasing with increasing external $[K^+]$ or progressive cell depolarization (Fig. 8). Contrary to the expectations, however, in the presence of a valinomycin-induced cell hyperpolarization, the activity of System X^-_{AG} decreased significantly (Table IV). Since a low concentration of valinomycin (enough to abolish the electrical potential across the mitochondrial membrane, but unable to hyperpolarize the cell, see Fig. 5) was effective in depressing amino acid transport through system X^-_{AG} comparably in polarized (low-K^+ medium) and in depolarized (high-K^+ medium) cells (see Table V), it is likely that this K^+ ionophore inhibits the activity of this system by a mechanism independent of the membrane potential.

A potential-sensitive, Na^+-independent system resembling system y^+ has been described in vesicles from brush-border membranes of cortex kidney cells [33]. Our studies indicate that system y^+ is indeed dependent upon the membrane potential, its activity being lower in depolarized cells (Table II) and higher in hyperpolarized cells (Table IV). The marked stimulatory effect of intracellular amino acids on L-arginine uptake by fibroblasts (trans-stimulation of inward transport) decreases the apparent sensitivity of system y^+ to the membrane potential (Table III). This suggests that the occupied form of the carrier is neutral (thus being little influenced by the membrane potential), while the empty form negatively charged.

Finally the two Na^+-independent systems L and x^-_C proved to be unaffected by changes in the external K^+ concentration (Tables II and IV). They are, therefore, independent of the membrane potential. This is not surprising for system L which acts chiefly in exchange, translocating neutral substrates in the absence of any transfer of net charges across the plasma membrane. The lack of potential dependence exhibited by system x^-_C was more surprising since this system carries amino acids in anionic form [2]. The absence of membrane potential dependence suggests that system x^-_C does not act to transfer net charge.

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