Changes in Membrane and Surface Potential Explain the Opposite Effects of Low Ionic Strength on the Two Lysine Transporters of Human Erythrocytes*

(Rosa Devés‡ and Sylvia Angelo
From the Department of Physiology and Biophysics, Faculty of Medicine, University of Chile, Santiago, Chile)

Several transport systems for cationic permeants have been proposed to contain an Na+-binding region within the substrate site. These include the cationic amino acid transporter (system y+) in human erythrocytes (1), the broad-scope amino acid transporter (b0,+), and the choline transporter in mouse blastocysts (2, 3). This hypothesis was postulated to explain the increase in the rate of entry that occurs when the standard NaCl medium is replaced by isotonic sucrose and the reduction in the apparent Kₘ for the cationic substrate that results from the same substitution. In erythrocytes, lysine uptake (5 μM, 20 °C) was found to be 3-fold higher in isotonic sucrose medium than in 150 mM NaCl, while the apparent Kₘ changed from 59 μM in NaCl to 15 μM in sucrose (1). Similarly, in blastocysts, Na+ replacement by uncharged osmolites induced an approximately 3-fold increase in lysine influx (0.42 μM, 37 °C), which was accompanied by a shift in apparent Kₘ from 61 to 5 μM (2).

The hypothesis stating that an Na+ binding site exists within the substrate site of cationic amino acid transporters has given rise to some considerations of general significance. It has been remarked that it is consistent with the proposal that the Na+-dependent interaction of neutral analogues with system y+ involves Na+ binding at the position otherwise taken by the distal cationic group of the basic amino acid (1). This idea was conceived 25 years ago by Christensen et al. (4, 5). In addition, the susceptibility of various transporters to Na+ inhibition (and/or their interaction with harmaline, a putative Na+ site ligand) has been used to draw a parallel among the structures of their substrate sites. It has been suggested that they might represent a family of closely related proteins with a common evolutionary origin. The family would include system y+, system b0,+ , the choline transporter, and the neutral amino acid transport systems ASC and asc; the last two are Na-dependent and Na-independent transport systems for alanine, serine, and cysteine, respectively (1–3, 6).

However, an inspection of the original data reveals that some observations are not compatible with a direct interaction between Na+ and the transporter. First, lysine influx in sucrose medium is also reduced by other monovalent cations, e.g. K+, Rb+, Li+, choline, and Tris+; their effects occur in a similar concentration range (IC₅₀ of approximately 25 mM) and with analogous kinetics (1–3). It is difficult to imagine a binding site that does not distinguish among such a variety of different cations. Second, inhibition appears to be partial, leveling off at approximately 25–30% of the original flux (1, 2); this is inconsistent with the behavior of a competitive inhibitor that should produce complete inhibition at sufficiently high concentrations. Therefore, alternative explanations for the mechanism of action of Na+, other than direct binding at the substrate site, should be considered.

Since the previous results were reported, lysine uptake into human erythrocytes has been shown to occur through two separate transporters, differing in their affinities for lysine and their ability to recognize neutral amino acids (7). System y+ has a lower affinity (Kₘ,y+ 109 μM), and its specificity is restricted to cationic amino acids; system y′L recognizes lysine with higher affinity (Kₘ,y′L 10 μM) and can also function in the presence of Na+ as a carrier for neutral amino acids. At low substrate concentrations, both systems contribute about equally to influx, but at high concentrations, the activity of system y′, which has a 10-fold higher Vₘₐₓ exceeds that of system yL (8, 9). It is not known which of the two systems is responsible for the Na+ effects reported earlier (1).

With the aim of reexamining the effect of monovalent cations upon the mediated transport of cationic permeants, we have investigated the effect of low ionic strength on the influx of lysine into human erythrocytes separating the contribution of the two transporters (8).

**EXPERIMENTAL PROCEDURES
Materials—Uniformly labeled L-[14C]lysine was purchased from Amersham (approximately 12 GBq mmol⁻¹); unlabeled amino acids, vali-

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‡ Dept. de Fisiología y Biofísica, Facultad de Medicina, U. de Chile, Casilla 70005, Santiago 7, Chile. Tel.: 562-6786314; Fax: 562-7776916; E-mail: rdeves@bitmed.med.uchile.cl.

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nomycin, and NEM were from Sigma; and dibutylphthalate was from Merck. Other chemicals were of commercial reagent grade.

Preparation of Cells—Human blood was obtained fresh from donors, using heparin as an anticoagulant, and erythrocytes were isolated as reported (7–9). Cells were washed in 5 mM sodium phosphate buffer (pH 6.8), containing 150 mM NaCl and 4 mM KCl. The same solution was used in an overnight incubation (to reduce endogenous amino acids), but the pH varied depending on the experiment (6.8, 7.2, or 7.45) as indicated below.

Treatment with NEM—The procedure used has been described elsewhere (8, 9). Washed erythrocytes were incubated with NEM (0.2 mM, 25°C, 15 min) in 5 mM sodium phosphate buffer plus 150 mM NaCl and 4 mM KCl. The pH of the solution was the same as the pH used in the overnight incubation (6.8, 7.2, or 7.45) as indicated below.

Measurement of Entry Rates—Influx was measured by following L-[14C]lysine uptake as previously reported (8). Each rate was estimated from linear regression analysis of at least 6 time points. Since several experiments were carried out under conditions that produce strong depolarization, which in turn affects the H+ distribution (causing acidification of the extracellular medium), an experimental strategy was devised to maintain the external pH constant at 6.8 during the uptake assay. Cells that were to be assayed under depolarizing conditions (e.g. with isotonic sucrose or sodium glucuronate) were incubated overnight at pH 7.45. The cells were then added to the corresponding assay medium (pH 7.45), and influx was initiated after the H+ distribution had reached equilibrium (10 min).

In experiments involving different concentrations of chloride, the cells were incubated overnight in solutions containing 150 mM NaCl, 4 mM KCl, and sodium phosphate, and the pH was adjusted to different values, depending on the chloride concentration to be used in the assay (0–15 mM Cl\(^-\), pH 7.45; 20–40 mM Cl\(^-\), pH 7.2; 75–145 mM Cl\(^-\), pH 6.8). The cells were subsequently suspended in solutions buffered with 5 mM MOPS at the same pH used in the overnight incubation, and the assay was started after 10 min. In all cases, the external pH during the uptake assay was approximately 6.8.

Appropriate controls showed that influx through systems y' and y'\(^+\)L is insensitive to changes in pH in the range 6.8–7.45. Cells were incubated overnight at pH 6.8 and 7.45, and the rate of entry was measured at the corresponding pH. No significant differences in the activities of these transporters were observed in the two groups of cells (results not shown).

RESULTS

The experiment shown in Fig. 1 compares the rates of lysine uptake into human erythrocytes suspended in NaCl or sucrose medium. Initial entry rates were measured in intact and NEM-treated cells in order to separate the contributions of system y' and system y'\(^+\)L. As reported earlier (1), the total lysine influx measured at low substrate concentrations increased when the NaCl medium was replaced by sucrose. Interestingly, however, activation resulted from a selective stimulation of system y'\(^+\)L (NEM-insensitive), whereas the activity of system y' (NEM-insensitive) remained unchanged.

\(^1\) The abbreviations used are: NEM, N-ethylmaleimide; MOPS, 4-morpholinopropanesulfonic acid.
selective increase of the activity of system $y^+$ was induced by addition of 2 mM valinomycin in the presence of a K$^+$ gradient. Uptake in the presence (●) or absence (△) of valinomycin (Val) was measured in intact (panel A) and NEM-treated cells (panel B). The calculated entry rates via system $y^+$ (NEM-sensitive) and system $y^-$ (NEM-insensitive) are shown in the insert. External medium contained 5 mM MOPS/Na$^+$ (pH 6.8), 140 mM NaCl, 4 mM KCl, and $t$-$[14C]$lysine (1 mM). Valinomycin was added 15 s before starting the assay.

Table I

Effect of valinomycin on the activity of system $y^+$ and system $y^-$ in the presence or absence of an outwardly directed K$^+$ gradient

| Experiment | System | $K_{cat}$, 4 mM | Rate ($\mu$moles/liter cell/min) |
|------------|--------|----------------|---------------------------------|
|            |        | $+Val$         | $-Val$                          |
| 1          | $y^+$  | 0.222 ± 0.008  | 0.046 ± 0.007                   |
|            | $y^-L$ | 0.033 ± 0.004  | 0.023 ± 0.002                   |
| 2          | $y^+$  | 0.435 ± 0.017  | 0.065 ± 0.010                   |
|            | $y^-L$ | 0.057 ± 0.016  | 0.022 ± 0.001                   |

The membrane potential dependence of lysine uptake was further investigated by producing the opposite shift in membrane potential. Membrane depolarization was attained by replacing external chloride with the impermeant anion glucuronate (10, 12). As expected from the previous experiment, total lysine flux was reduced substantially, whereas the NEM-resistant flux was only slightly affected (Fig. 4). Therefore, only system $y^-$ was influenced by membrane potential to a significant degree. In five identical experiments, the substitution of chloride with glucuronate reduced the rate of system $y^-$ by 85 ± 4.7% (S.E.) and the rate of system $y^-L$ by 17 ± 4.4%.

The observation that influx through system $y^-L$ is independent of membrane potential, indicates that the activation observed in sucrose medium must result from Na$^+$ removal per se. Fig. 5A shows the effect of increasing Na$^+$ concentrations on the rate of lysine entry into NEM-treated erythrocytes suspended in sucrose. The rate was seen to diminish with increasing Na$^+$ concentration, as reported by other authors for the total lysine flux (1). The effect was also observed for other monovalent cations such as K$^+$, Li$^+$, and choline (panel B). The dependence between rate and concentration can be best described by an exponential function assuming partial inhibition.

Divalent cations (Mg$^{2+}$, Ca$^{2+}$) were found to produce the same effect as monovalent cations but at substantially lower concentrations (Fig. 6). The effect of mobile ions is consistent with the hypothesis that ions are affecting the surface potential of the cells (13–15). Guanidinium ion (a substrate analogue)
also inhibited lysine entry, but in this case, complete inhibition was obtained at sufficiently high concentrations.

Finally, if the above conclusions are correct, the stimulatory effect of sucrose should disappear at high substrate concentrations because, under these conditions, cationic amino acid influx into human erythrocytes occurs mainly via system y\textsubscript{1} (8). Moreover, at the higher substrate concentrations, lysine influx should not be significantly affected by the surface potential (under “Discussion”) (15). The experiments depicted in Fig. 7 show that, as predicted, the total fluxes of arginine (100 mM) and lysine (50 mM) were found to decrease when NaCl was replaced by sucrose.

**DISCUSSION**

The results reported here show that the increase of lysine influx, observed when human erythrocytes are suspended in sucrose, is due to the activation of lysine entry via system y\textsuperscript{1}L. The activity of system y\textsuperscript{1}, the other cationic amino acid transporter present in these cells, is reduced by the same maneuver. In neither case are the observed effects compatible with a direct
interaction between Na\textsuperscript{+} and the transporter, as previously proposed (1), but they are the result of changes in the surface potential (system y\textsuperscript{+L}) and membrane potential (system y\textsuperscript{+}) of the cells.

The effect of membrane potential on the activity of system y\textsuperscript{+L} in human erythrocytes is consistent with observations made in other experimental systems. Transport of lysine and arginine through the murine cationic amino acid transporter (mCAT-1), expressed in Xenopus laevis, has been shown to be electrogenic (16). It has been proposed that the effect of voltage on the steady-state transport rate arises, 1) from charge movement across the membrane field during the conformational transition of the free carrier and 2) from an “ion well” effect that influences binding. Similar conclusions were reached in studies with placental brush-border membranes (17).

The lack of effect of the membrane potential on the activity of system y\textsuperscript{+L} indicates that, under the present experimental conditions, the rate-limiting step in this process does not involve charge movement. This finding is in agreement with our previous proposal that the broad scope, high affinity system y\textsuperscript{+L} participates in the exchange of lysine for endogenous neutral amino acids plus sodium (9).

The other conclusion of this work states that the sucrose-induced stimulation of lysine influx via system y\textsuperscript{+L} can be accounted for by changes in the surface potential and is unrelated to a direct interaction between Na\textsuperscript{+} and the carrier.

Human erythrocytes have a net negative surface charge ($2 \times 10^{-7}$ fixed negative charges in an area of 140 $\mu$m$^2$) and consequently give rise to a surface potential whose value depends upon the ionic strength of the surrounding medium (13, 18). According to the Boltzmann distribution of ions in an electric field, such a potential creates a difference between the concentration of ions in the bulk phase and at the membrane-solution interface. The negative charges on the membrane surface attract cations and repel anions, and a balance is established between the attractive forces generated by the electrostatic field and the statistical tendency of the counterions to diffuse away from the surface at which they are concentrated. Thus, changes in surface potential are expected to influence the flux of ionic substrates by affecting the substrate concentration near the membrane surface (14, 15, 19).

According to the Gouy-Chapman equation, the surface potential is dependent on the concentration of mobile ions in the bulk solution; counterions exert a “screening” effect reducing the surface potential. Multivalent counterions are more effective than univalent ones and, unless there are specific binding sites for some ions, the effect is independent of the nature of the ion. Divalent cations are expected to be approximately one order of magnitude more effective in changing the negative surface potential than the monovalent ions (14, 19).

The influence of surface charge on the kinetics of ion-translocation across biological membranes has been theoretically...
analyzed by Theuvenet and Borst-Pauwels (15). The analysis predicts that the stimulatory effect of a negative surface potential upon the influx of a cationic substrate depends on the substrate concentration. The effect is expected to diminish as the substrate concentration is raised and should disappear when the system has reached saturation. Since increasing the salt concentration in the bulk solution reduces the surface potential, mobile cations will inhibit influx with an apparent competitive behavior. However, inhibition is expected to be partial because the effect of mobile ions should lessen as the surface potential approaches zero (15).

The inhibition of lysine influx by monovalent and divalent ions described here is entirely consistent with the considerations stated above. 1) Different monovalent cations (Na\(^+\), K\(^+\), Li\(^+\), and choline) inhibited lysine influx (1 \(\mu\)M) with similar concentration dependence. 2) Inhibition reached a maximum when the flux approached 35–40% of the original value. 3) The magnitude of the observed effect and the concentration dependence are both consistent with the interpretation. The surface potential of human erythrocytes, suspended in solutions containing 150 mM NaCl or 10 mM NaCl, have been calculated to be near -2.3 mV and -2.25 mV, respectively (13, 18). The predicted ratio for the fluxes, measured in the two media, is thus 2.55. This value is in good agreement with the results shown in Figs. 5 and 6. In addition, the fact that the surface potentials estimated at 100 and 150 mM NaCl do not differ significantly (13) is consistent with the concentration dependence observed. 4) Divalent cations were also partial inhibitors of lysine entry, but the effect occurred at considerably lower concentrations. 5) Guanidinium ion, an analogue of arginine, showed a different inhibition pattern producing complete inhibition at sufficiently high concentrations, as expected from a competitive ligand. 6) The sucrose-induced stimulation was either abolished or substantially reduced at high substrate concentrations (Fig. 7).

In spite of the above conclusions, the direct interaction between Na\(^+\) and the transporter may explain other properties of system y'. In fact, binding of neutral amino acids to this transporter has been found to be dramatically influenced by the ionic composition of the medium; the \(K_i\) for leucine is 4, 10, and 700 \(\mu\)M, in Li\(^+\), Na\(^+\), or K\(^+\), respectively (8, 9). The potentiation of leucine binding by Na\(^+\) or Li\(^+\) probably involves the interaction between the transporter and the cation at a relevant site.

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REFERENCES
1. Young, D. J., Fincham, D. A., and Harvey, C. M. (1991) Biochim. Biophys. Acta 1070, 111–118
2. Van Winkle, L. J., Campione, A. L., and Gorman, J. M. (1990) Biochim. Biophys. Acta 1025, 215–224
3. Van Winkle, L. J., Campione, A. L., Mann, D. F., and Wasserlauf, H. G. (1993) Biochim. Biophys. Acta 1146, 38–44
4. Christensen, H. N., Handlogten, M. E., and M. Thomas, E. L. (1969) Proc. Natl. Acad. Sci. U.S.A. 63, 948–955
5. Thomas E. L., Shao, T.-C., and Christensen, H. N. (1971) J. Biol. Chem. 246, 1677–1681
6. Van Winkle, L. J. (1993) Biochim. Biophys. Acta 1154, 157–172
7. Deves, R., Chavez, P., and Boyd, C. A. R. (1992) J. Physiol. (Lond.) 454, 491–501
8. Deves, R., Angela, S., and Chavez, P. (1993) J. Physiol. (Lond.) 468, 753–766
9. Angela, S., and Deves, R. (1994) J. Membr. Biol. 141, 183–192
10. Bernhardt, I., Hall, A. C., and Ellory, J. C. (1991) J. Physiol. (Lond.) 434, 489–506
11. Rink, T., and Hladky, S. B. (1982) in In Red Cell Membranes: A Methodological Approach (Ellory, J. C., and Young, J. D. eds) pp. 321–333, Academic Press, London
12. Freedman, J. C., and Hoffman, J. F. (1979) J. Gen. Physiol. 74, 187–212
13. Heinrich, R., Gaestel, and Glaser, R. (1981) Acta Biol. Med. Ger. 40, 765–770
14. McLaughlin, S. (1977) Curr. Top. Membr. Transp. 9, 71–144
15. Theuvenet, A. P. R., and Borst-Pauwels, W. F. H. (1976) J. Theor. Biol. 57, 313–329
16. Kavanagh, M. P. (1993) Biochemistry 32, 5781–5785
17. Elezo, N., Deves, R., and Boyd, C. A. R. (1994) J. Physiol. (Lond.) 479, 291–300
18. Bernhardt, I. (1994) in Cell Electrophoresis (Bauer, J., ed) pp. 163–179, CRC Press, Boca Raton, FL
19. Hille, B. (1990) Ionic Channels of Excitable Membranes, 2nd Ed., pp. 318–326, Sinauer Associates, Inc., Sunderland, Mass