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Increase of Na^+ Gradient-dependent L-Glutamate and L-Aspartate Transport in High K^+ Dog Erythrocytes Associated with High Activity of (Na^+,K^+)-ATPase*

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As reported previously, some dogs possess red cells characterized by low Na^+, high K^+ concentrations, and high activity of (Na^+,K^+)-ATPase, although normal dog red cells contain low K^+, high Na^+, and lack (Na^+,K^+)-ATPase. Furthermore, these red cells show increased activities of L-glutamate and L-aspartate transport, resulting in high accumulations of such amino acids in their cells. The present study demonstrated: (i) Na^+ gradient-dependent L-glutamate and L-aspartate transport in the high K^+ and low K^+ red cells were dominated by a saturable component obeying Michaelis-Menten kinetics. Although no difference of the K_m values was observed between the high K^+ and low K^+ cells, the V_{max} values for both amino acids' transport in the high K^+ cells were about three times those of low ones. (ii) L- and D-aspartate, but not D-glutamate, competitively inhibited L-glutamate transport in both types of the cells. (iii) Ouabain decreased the uptake of the amino acids in the high K^+ dog red cells, whereas it was not effective on those in the low K^+ cells. (iv) The ATP-treated high K^+ cells ($[K^+]_i \fallingdotseq [K^+]_o$, $[Na^+]_i > [Na^+]_o$) showed a marked decrease of both amino acids' uptake rate, which was almost the same as that of the low K^+ cells. (v) Valinomycin stimulated the amino acids' transport in both of the high K^+ and the ATP-treated low K^+ cells ($[K^+]_i > [K^+]_o$, $[Na^+] < [Na^+]_o$), suggesting that the transport system of L-glutamate and L-aspartate in both types of the cells might be electrogenic. These results indicate that the increased transport activity in the high K^+ dog red cells was a secondary consequence of the Na^+ concentration gradient created by (Na^+,K^+)-ATPase.

Although dog erythrocytes have been known to contain low K^+ and high Na^+ concentrations and to lack the membrane bound enzyme (Na^+,K^+)-ATPase (1, 2), we have recently found the presence of (Na^+,K^+)-ATPase and high K^+, low Na^+ concentrations in the erythrocytes of some dogs associated with hereditary high concentrations of reduced glutathione and some amino acids, glutamate, aspartate, and glutamine in their cells (3). The accumulation of GSH, which was 5–7 times the normal concentration, could be explained by the fact that feedback inhibition of γ-glutamylcysteine synthetase by GSH was released by the high level of glutamate (about 92 times the normal) in their cells (4). We have also found that L-glutamate and L-aspartate uptake by these cells was greatly increased, and that the uptake was saturable and entirely consistent with simple Michaelis-Menten kinetics.

Dog erythrocytes possess a high affinity, Na^+-dependent transport system for L-glutamate and L-aspartate (5), although most mammalian erythrocytes are impermeable to them (6). The L-glutamate and L-aspartate transport system is also present in other tissues such as the renal proximal tubule (7–9), liver (10), and the central nervous system (11–15). In recent years, a high affinity Na^+-dependent transport system for anionic amino acids, designated as System X^-_{AG}, has been shown in the cultured rat hepatocytes and human skin fibroblasts (16, 17). Thus, it has been considered that L-glutamate and L-aspartate are transported by a high affinity, Na^+ gradient-dependent system. Furthermore, the K^+ gradient is also thought to play a role in the transport of L-glutamate and L-aspartate into the renal brush-border membrane vesicles (7, 9) and into rat cerebral cortex synaptosomes (15). In addition, ouabain, which inhibits (Na^+,K^+)-ATPase specifically, reduced synaptosomal uptake of L-glutamate (13) and various neurotransmitters (18, 19). These results have led to the suggestions that the Na^+ and K^+ gradient across the membrane may provide the driving force for L-glutamate and L-aspartate transport into such cells or vesicles. The mechanism for creating the ion gradient involves (Na^+,K^+)-ATPase (10, 20). Thus, in a previous report we concluded that the Na^+ and K^+ gradient created by the function of (Na^+,K^+)-ATPase accelerated the transport of L-glutamate and L-aspartate into dogs' erythrocytes with high K^+, low Na^+ concentrations (high K^+ cells), which might result in a high accumulation of such amino acids in the cells (3).

In the present study, we examined the characteristics of the Na^+ gradient-dependent L-glutamate and L-aspartate transport system in high K^+ erythrocytes, and clearly demonstrated that the increased transport activity in these cells is a secondary consequence of a Na^+ concentration gradient induced by (Na^+,K^+)-ATPase, and that the amino acids' transport system in dog erythrocytes is electrogenic.

EXPERIMENTAL PROCEDURES

Materials—L-[2,3-^{3}H]Glutamate (21.6 Ci/mmol) and L-[2,3-^{3}H]aspartate (14.3 Ci/mmol) were purchased from New England Nuclear. The sources of other materials used in this work were as follows: valinomycin from P-L Biochemicals., ouabain from Merck, and Na_2ATP from Boehringer Mannheim. All other reagents used were from Wako Pure Chemical Industries Ltd. (Osaka, Japan), or from Dojindo Laboratories (Kumamoto, Japan).

Preparation of Erythrocyte Suspensions—Preparation of erythrocytes was carried out as previously described (3) with some modification. Venous blood from each dog was heparin-treated and washed three times at 4 °C with 5 volumes of the incubation medium. Unless

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otherwise indicated, the incubation medium contained 150 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 10 mM D-glucose, 0.1% (w/v) BSA[1] (fraction V), and 15 mM MOPS/NaOH (pH 7.5). The washed cells were resuspended in the same medium to yield hematocrit values of 15–20% and kept at 4 °C until used (approximately 30 min). When the uptakes were measured using an Na^+-free medium, MOPS/Tris (pH 7.5) was used instead of MOPS/NaOH, and choline chloride was substituted for NaCl.

Transport Experiments—The transport experiment was carried out by the method of Ellory *et al.* (5) with a slight modification. The erythrocyte suspension and the medium containing appropriate labeled and nonlabeled amino acids were kept at 37 °C for 5 min. After that, amino acids' uptake by the cells was measured at 37 °C by mixing 0.15 ml of the cell suspension with 0.15 ml of the medium containing the appropriate radioactive amino acids (0.5–5 μCi/ml) and the appropriate concentration of each of the nonlabeled amino acid (0.5–500 μM). Incubations were stopped by the addition of 1.0 ml of the ice-cold BSA- and amino acid-free medium. Erythrocytes were rapidly washed three times with 1.0 ml of the ice-cold medium by centrifugation at 15,000 × *g* for 10 s using a microcentrifuge MC-15A (Tomy Seiko Co., Ltd., Japan), and then the packed cells were lysed with 0.5 ml of 0.5% (v/v) Triton X-100 in water. The hemolysate was deproteinized by the addition of 0.5 ml of 5% (w/v) trichloroacetic acid, and centrifuged at 15,000 × *g* for 3 min. Nine hundred microliters of the protein-free supernatant were added to 10 ml of Scintizol EX-H (Wako Pure Chemical Industries, Ltd., Japan) in a scintillation vial, and counted for radioactivity by a liquid scintillation spectrometer LS-500 (Horiba Ltd., Japan).

Preliminary time course experiments in which incubations were stopped at the selected time intervals (10 s to 60 min) showed that the amino acid uptake was almost linear for the initial 60 s in each of the concentrations of extracellular amino acids. Thus, we expressed the amino acids' uptakes as the initial uptake rates, *i.e.* the flux in the initial 60 s (μmol/liter of RBC/min). Under these conditions, the intracellular accumulations of labeled amino acids did not exceed 10% of the initial extracellular levels.

In addition, in the preliminary experiments we found that the addition of BSA into the incubation medium at a concentration of 0.1% (v/v) inhibited hemolysis completely, even over long time incubation (60–120 min). Although Rhoads *et al.* (21) reported that BSA at this concentration reversed the inhibition of the synaptosomal Na^+-dependent proline uptake by monosaturated fatty acids, the amino acid uptake by the dog erythrocytes was not changed by the addition of BSA into the incubation medium (data not shown).

Ouabain Treatment of Dog Erythrocytes—Ouabain, a cardiac glycoside which specifically inhibits the activities of (Na^+,K^+)-ATPase, was used to determine the relationship between the transport of L-glutamate and L-aspartate and the function of (Na^+,K^+)-ATPase in high K^+ cells.

Ouabain was added to the erythrocyte suspensions at a final concentration of 0.1 mM, which was a sufficient amount to inhibit (Na^+,K^+)-ATPase activities in high K^+ dog erythrocyte membrane preparations as described in the previous report (3). Incubations were carried out for the selected time at 37 °C with gentle shaking, and then L-glutamate and L-aspartate uptakes were examined as mentioned above.

Estimation of the Effect of Valinomycin-induced Membrane Potential on L-Glutamate and L-Aspartate Uptake—Valinomycin in absolute ethanol (1 mg/ml) was added to the medium containing labeled and nonlabeled amino acids to give a concentration of 6.7 μg/ml for valinomycin. As a control experiment, an equal volume of absolute ethanol was added to the medium. Under these conditions, valinomycin and ethanol were contained at the final concentrations of 3.4 μg/ml and 0.34% (v/v), respectively, in the transport experimental system.

Exchange of Intracellular Na^+ and K^+ Concentration by ATP—To examine the effect of Na^+ and K^+ concentrations on the transport of amino acids in the cells, intracellular Na^+,K^+ concentrations were changed by the addition of ATP in an incubation medium. It has been known that dog erythrocytes become permeable to Na^+ and K^+ when they are placed in media containing ATP in concentrations of less than 0.5 mM (22, 23).

The ATP treatment of dog erythrocytes was carried out by the method of Parker and Snow (22) with a minor modification. All the procedures were carried out at room temperature unless otherwise indicated. Heparin-treated dog blood was washed three times with a medium containing 150 mM NaCl (or KCl), 5 mM KCl (or NaCl), 10 mM D-glucose, 0.05% (w/v) BSA, and 15 mM MOPS/Tris (pH 7.5), and then suspended in the same medium to yield a hematocrit value of 7–8%. ATP was added to the suspension at a final concentration of 1.0 mM. The cells were incubated for 1 h at 37 °C with gentle shaking and then washed twice with the same medium without ATP. After that, the cells were washed more than three times with the medium for transport experiments. The washed cells were resuspended in the medium for the transport experiment to yield to hematocrit value of 15–20%, and the amount of amino acid uptake by the cells was measured, as mentioned above, within 30 min after the preparation of these suspension. Simultaneously, the intracellular Na^+ and K^+ concentrations of these cells were rapidly estimated using a flamephotometer 205D (Hitachi Ltd., Japan), as reported previously (3).

The effect of valinomycin-induced membrane potential on L-glutamate and L-aspartate transport in the dog erythrocytes treated or not treated with ATP were examined as described above.

RESULTS

Effect of Na^+ on L-Glutamate and L-Aspartate Transport in High K^+ and Low K^+ Erythrocytes in Dogs—Ellory *et al.* (5) demonstrated that L-glutamate and L-aspartate uptake into normal dog erythrocytes (low K^+ erythrocytes) had high affinity and Na^+-dependent components over a substrate concentration range of 1–20 μM. We examined the Na^+ gradient-dependent uphill transport of L-glutamate and L-aspartate into high K^+ erythrocytes in comparison with that in low K^+ cells over a wider range of 0.5–500 μM (physiologically about 100 μM for glutamate and almost 0 for aspartate in the plasma). Fig. 1*a* shows the initial uptake rates of L-glutamate into high K^+ and low K^+ cells in the presence or absence of Na^+ in the medium. When Na^+ was present in the medium, the initial uptake rates of L-glutamate into the high K^+ erythrocytes were approximately 5 (at the extracellular L-glutamate concentration of 0.5 μM) to 1.7 (at 500 μM) times those of the low K^+ cells. The uptake rates of both high K^+ and low K^+ cells under the condition of absent Na^+ were linear in each of the concentrations of extracellular amino acids and almost the same throughout the concentration range

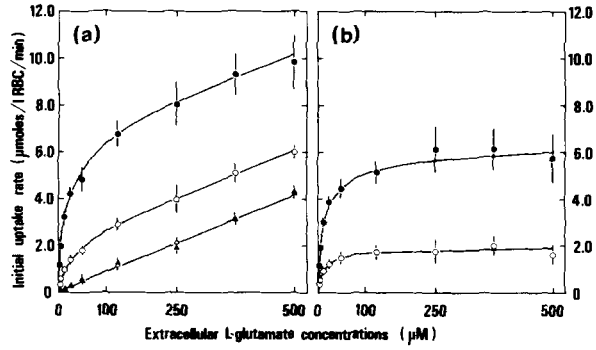

FIG. 1. **Effect of Na^+ on L-glutamate transport in high K^+ and low K^+ dog erythrocytes.** *a*, L-glutamate transport in the presence (● and ○) or absence (▲ and △) of extracellular Na^+. ● and ▲, high K^+ dog erythrocytes ($n = 8$); ○ and △, low K^+ dog erythrocytes ($n = 7$). Uptakes were measured at 37 °C for 60 s in the medium containing 150 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 10 mM D-glucose, 0.1% (w/v) BSA, and 15 mM MOPS/NaOH (pH 7.5) when Na^+ was present. Choline chloride and MOPS/Tris (pH 7.5) were substituted for NaCl and MOPS/NaOH (pH 7.5), respectively, when Na^+ was absent. The detailed procedures of the L-glutamate uptake measurement are described under "Experimental Procedures." Data are expressed as mean ± S.D. *b*, Na^+ gradient-dependent L-glutamate uptake in high K^+ (●) and low K^+ (○) dog cells. Data were obtained by subtracting the uptake rate in the absence of Na^+ from the uptake rate in the presence of Na^+ at each concentration of L-glutamate, and expressed as mean ± S.D.

[1] The abbreviations used are: BSA, bovine serum albumin; MOPS, 3-(*N*-morpholino)propanesulfonic acid; RBC, red blood cells.

of 0.5–500 μM. The Na^+ gradient-dependent component, given by subtraction of the values in the absence of Na^+ from those in the presence of Na^+, was shown in Fig. 1*b*, indicating that the Na^+ gradient-dependent uptake was dominated by a saturable component (saturated at about 125 μM) obeying Michaelis-Menten kinetics. This Na^+ gradient-dependent L-glutamate transport in the high K^+ erythrocytes showed a marked increase which was three to four times that of the low K^+ cells. The K_m and V_{max} values from kinetic analysis of the Na^+ gradient-dependent L-glutamate transport in both types of cells are shown in Table I. No significant difference between the high K^+ and low K^+ erythrocytes was observed in the K_m values for L-glutamate, but the V_{max} value for L-glutamate transport in the high K^+ cells was about three times that of the low K^+ cells. The values of the kinetic constants from the low K^+ cells were slightly higher than those previously reported by Ellory *et al.* (5).

L-Aspartate transport in both types of cells showed a somewhat similar trend (Fig. 2, *a* and *b*, and Table I); however, L-glutamate transport was about twice that of L-aspartate transport in both type of erythrocytes.

Cross-inhibition Experiments of D- and L-Aspartate, D-Glutamate, and L-Glutamine on Na^+ Gradient-dependent L-Glutamate Transport in High K^+ and Low K^+Erythrocytes—Cross-inhibition experiments of D- and L-aspartate, L-glutamine, and D-glutamate on L-glutamate uptake by high K^+ and low K^+ cells were carried out and the results were given in Table II. The presence of 25 μM L-aspartate in the medium caused the increase of apparent K_m values for L-glutamate uptake by both types of cells with K_i values close to those of L-aspartate, and had no effect on V_{max} values; that is L-aspartate inhibited Na^+ gradient-dependent L-glutamate transport in a competitive manner. Moreover, D-aspartate at a concentration of 25 μM was also found to be a competitive inhibitor of L-glutamate transport in both types of cells, while the K_i value was about six times that of L-aspartate. In contrast to D- and L-aspartate, L-glutamine and D-glutamate had no effect on either of the apparent K_m and V_{max} values for L-glutamate transport at a concentration of 25 and 50 μM, respectively.

The inverse relationship (*i.e.* inhibitory effect of L-glutamate on L-aspartate uptake) was also seen (shown in Table II).

Effect of Ouabain on Na^+ Gradient-dependent L-Glutamate and L-Aspartate Transport in High K^+ and Low K^+ Erythrocytes—In this experiment, ouabain was used at a final concentration of 0.1 mM, which sufficiently inhibited the activity of (Na^+,K^+)-ATPase in the high K^+ erythrocyte membrane preparation as reported previously (3). We determined in a

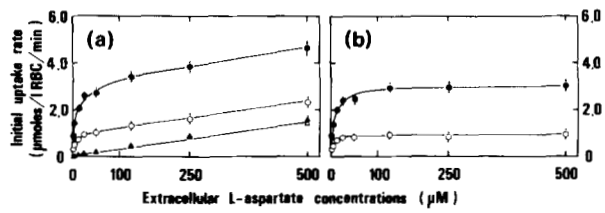

FIG. 2. **Effect of Na^+ on L-aspartate transport in high K^+ and low K^+ dog erythrocytes.** *a*, L-aspartate transport in the presence (● and ○) or absence (▲ and △) of extracellular Na^+. ● and ▲, high K^+ dog erythrocytes ($n = 8$); ○ and △, low K^+ dog erythrocytes ($n = 7$). Uptake was measured as described in the legend to Fig. 1*a* and under "Experimental Procedures." Data are expressed as mean ± S.D. *b*, Na^+ gradient-dependent L-aspartate uptake in high K^+ (●) and low K^+ (○) dog cells. Data were obtained as mentioned in the legend to Fig. 1*b* and expressed as mean ± S.D.

TABLE I

Kinetic constants for Na^+ gradient-dependent L-glutamate and L-aspartate transport in high K^+ and low K^+ dog erythrocytes

The procedures for the measurement of L-glutamate and L-aspartate transport were described under "Experimental Procedures" and in the legends of Figs. 1 and 2. These values were calculated from the double reciprocal plot.

Subjects	L-Glutamate		L-Aspartate	
	K_m	V_{max}	K_m	V_{max}
	μM	μmol/liter RBC/h	μM	μmol/liter RBC/h
High K^+ RBC ($n = 8$)	11.47 ± 1.55	390.6 ± 55.7	6.26 ± 0.23	180.9 ± 10.6
Low K^+ RBC ($n = 7$)	12.59 ± 1.74	114.4 ± 15.4	5.96 ± 0.76	64.1 ± 11.2

TABLE II

Effect of D- and L-aspartate, D-glutamate, and L-glutamine on Na^+ gradient-dependent L-glutamate transport in high K^+ and low K^+ dog erythrocytes

Incubations were started by the addition of the medium containing L-[^{3}H]glutamate, the appropriate concentration of nonlabeled L-glutamate, and the inhibitor to the erythrocyte suspensions. All other procedures are described under "Experimental Procedures." Data are expressed as mean values of the two experiments for each type of cells.

Substrates	Inhibitors	High K^+ RBC ($n = 2$)			Low K^+ RBC ($n = 2$)		
		K_m	V_{max}	K_i	K_m	V_{max}	K_i
		μM	μmol/liter RBC/h	μM		μmol/liter RBC/h	μM
L-Glutamate	None	11.1	375.0	—[a]	10.0	90.0	—
	25 μM L-aspartate	66.7	402.0	5.0	58.8	96.0	5.1
	25 μM L-glutamine	11.1	375.0	—	9.8	90.0	—
	25 μM D-aspartate	20.0	408.0	31.2	17.2	100.2	34.7
	50 μM D-glutamate[b]	9.4	370.4	—	9.6	112.2	—
L-Aspartate	None	6.2	193.2	—	6.3	58.8	—
	25 μM L-glutamate	20.3	204.2	11.0	17.2	63.1	9.1
	125 μM L-glutamate	80.4	210.0	10.4	66.7	66.7	12.9

[a] No significant inhibition.

[b] Data from an experiment for each type of cells. The control values for K_m and V_{max} are 9.1 and 350.9 in high K^+ cells, and 10.5 and 118.1 in low K^+ cells, respectively.

preliminary study that the inhibitory effect of ouabain on L-glutamate transport in high K^+ cells was dependent on an incubation time at 37 °C, and that the most effective incubation time was a period exceeding 3 h (shown in Fig. 3). Following this recommendation, the erythrocyte suspensions were incubated for 3 h at 37 °C.

Fig. 4 shows the effect of ouabain on Na^+ gradient-dependent L-glutamate transport in both high K^+ and low K^+ erythrocytes. Ouabain treatment decreased L-glutamate uptake by the high K^+ erythrocytes to 39–46%, whereas it had no effect of L-glutamate uptake by the low K^+ erythrocytes. This result demonstrated that ouabain had an effect only on the V_{max} value and no effect on the K_m value for Na^+ gradient-dependent L-glutamate transport in the high K^+ cells.

Ouabain treatment also decreased the velocity of L-aspartate transport in the high K^+ cells to approximately 70%, whereas it did not change that of the low K^+ cells (data not shown).

These results strongly suggest that the ouabain-sensitive, (Na^+,K^+)-ATPase-dependent transport system for L-glutamate and L-aspartate may be present in the high K^+ erythrocytes but not in the low K^+ cells.

Effect of Valinomycin-induced Membrane Potential on Na^+ Gradient-dependent L-Glutamate and L-Aspartate Transport—From the result that the transport of L-glutamate and L-aspartate in both high K^+ and low K^+ cells was specifically enhanced in the presence of an Na^+ concentration gradient, it seems possible that the transport of the two amino acids in both types of the cells is coupled with the flux of Na^+ (Na^+ L-glutamate or Na^+ L-aspartate cotransport). The following experiment was therefore carried out to determine if the Na^+ amino acids cotransport system was electrogenic or electroneutral. Valinomycin was used in this experiment to create a diffusion potential across the cell membrane.

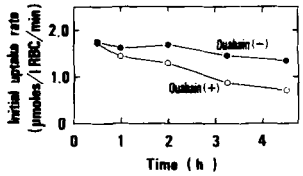

FIG. 3. **Incubation time-dependent inhibitory effect of ouabain on L-glutamate transport in high K^+ dog erythrocytes.** ●, incubated in the medium without ouabain; ○, incubated with 0.1 mM ouabain. Ouabain when present was added to the erythrocyte suspension at a concentration of 0.1 mM. Incubations were carried out for the appropriate time at 37 °C with a gentle shaking, and then L-glutamate uptake was measured at the extracellular nonlabeled L-glutamate concentration of 5 μM. In this experiment, the uptake was examined using only the medium containing 150 mM NaCl and 15 mM MOPS/NaOH (pH 7.5), since almost all of the uptake of L-glutamate at the extracellular concentration of 5 μM was Na^+ gradient-dependent (Fig. 1). Data are the mean values of two experiments.

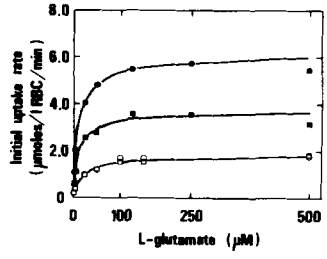

FIG. 4. **Effect of ouabain on Na^+ gradient-dependent L-glutamate transport in high K^+ (● and ■) and low K^+ (○ and □) dog erythrocytes.** L-Glutamate uptake was measured in each cell as described under "Experimental Procedures" after the incubation for 3 h at 37 °C with (■ and □) or without (● and ○) 0.1 mM ouabain. All values were obtained by subtracting the uptake rate in the absence of Na^+ from the uptake rate in the presence of Na^+, and are expressed as the mean values of two experiments.

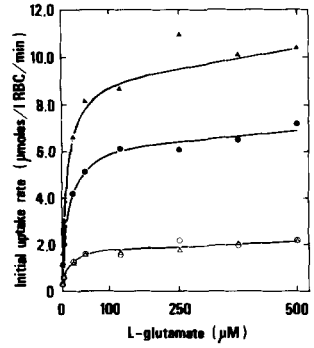

FIG. 5. **Effect of the membrane potential induced by valinomycin on Na^+ gradient-dependent L-glutamate uptake in high K^+ (● and ▲) and low K^+ (○ and △) dog erythrocytes.** Valinomycin in absolute ethanol (1 mg/ml) or only absolute ethanol were added to the medium at a final concentration of 3.4 μg/ (▲ and △) and 0.34% (● and ○), respectively. All values were obtained as described in the legend to Fig. 4, and expressed as the mean values of three experiments.

Fig. 5 shows the effect of valinomycin on Na^+ gradient-dependent L-glutamate transport in the high K^+ and low K^+ erythrocytes. It is clear that valinomycin markedly increased L-glutamate uptake by the high K^+ erythrocytes. The V_{max} value for L-glutamate uptake by the high K^+ cells in the presence of valinomycin was 1.5 times the value when valinomycin was absent, whereas the K_m value was not changed. On the contrary, valinomycin was not effective for L-glutamate transport in the low K^+ cells.

L-Aspartate transport in the high K^+ and low K^+ cells showed the same responses to valinomycin, respectively (data not shown).

These results indicate that the transport of L-glutamate and L-aspartate in the high K^+ erythrocytes is electrogenic.

Responses of L-Glutamate Transport to the Reversal of Intracellular Na^+ and K^+ Concentration by ATP Treatment and the Effect of Valinomycin on the Transport—From the above experiments we considered that the increased uptake of L-glutamate and L-aspartate by the high K^+ cells may be due to the large Na^+ and K^+ collective concentration gradient across the cell membrane ($[Na^+]_{out} > [Na^+]_{in}$, $[K^+]_{out} < [K^+]_{in}$), created by (Na^+,K^+)-ATPase of the high K^+ cells. If this idea is correct, the amino acids' uptake by the high K^+ cells will decrease if their intracellular Na^+ and K^+ concentrations are reversed ($[Na^+]_{in} > [K^+]_{in}$).

To check this assumption, we examined L-glutamate uptake by the high K^+ and low K^+ cells in which Na^+ and K^+ concentrations were reversed by ATP treatment, and we also examined the effect of valinomycin-induced membrane potential on the uptake.

Fig. 6*a* shows the Na^+ and K^+ concentrations present in high K^+ and low K^+ dog erythrocytes after incubation with or without 1 mM ATP at 37 °C for 1 h. The presence of ATP in the incubation medium resulted in complete reversal of concentration of Na^+ and K^+ in both types of cells, whereas the cells not treated with ATP maintained compositions of Na^+ and K^+ concentrations near their physiological levels during the incubation, respectively. Fig. 6*b* shows L-glutamate transport in the erythrocytes treated or not treated with ATP and the effect of valinomycin on the transport. The low K^+ cells

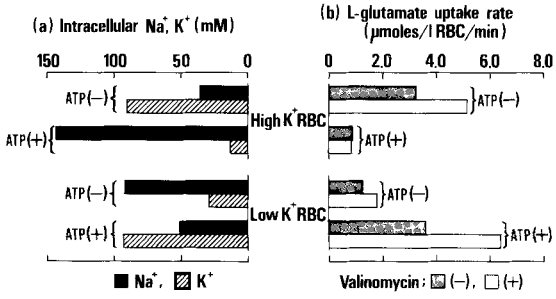

FIG. 6. **Effects of ATP on the intracellular electrolyte compositions and L-glutamate transport in dog erythrocytes, and the effect of valinomycin-induced membrane potential on the transport in the cells treated with ATP.** *a*, changes of Na^+ and K^+ concentrations in the dog erythrocytes treated with ATP. Washed high K^+ dog erythrocytes were incubated for 1 h at 37 °C in the medium containing 150 mM NaCl, 5 mM KCl, 10 mM D-glucose, 0.05% (w/v) BSA, 15 mM MOPS/Tris (pH 7.5), and 1 mM Na_2ATP when present. The low K^+ cells were incubated in the medium containing reversed concentrations of Na^+ and K^+ (5 mM NaCl, 150 mM KCl). After washing with the incubation medium with ATP, the cells were washed with the medium for the transport assay (as described in the legend to Fig. 1) three times and then resuspended to yield a hematocrit value of 15–20%. The uptake of L-glutamate by the cells was measured as described under "Experimental Procedures." Simultaneously, the intracellular concentrations of Na^+ and K^+ in the remaining cells were rapidly estimated as described under "Experimental Procedures." b, L-glutamate transport into the dog erythrocytes treated with ATP and the effect of valinomycin on the transport. L-Glutamate transport into the cells treated or not treated with ATP was measured at the extracellular L-glutamate concentration of 10 μM as described under "Experimental Procedures." Valinomycin was used at the final concentration of 3.4 μg/ml.

treated with ATP, in which the cation composition was similar to that of high K^+ dog erythrocytes, showed a marked increase of the uptake of L-glutamate, and the uptake rate was almost the same as that of the high K^+ cells not treated with ATP. Moreover, the L-glutamate uptake by the low K^+ cells treated with ATP was remarkably increased by the addition of valinomycin in the medium, as observed in the high K^+ erythrocytes not treated with ATP. On the contrary, the high K^+ cells treated with ATP, in which Na^+ was accumulated and K^+ was lost, showed a very low L-glutamate uptake rate which was not affected by valinomycin. The same results were obtained for L-aspartate uptake (data not shown).

These results clearly demonstrate that the transport of L-glutamate and L-aspartate in high K^+ cells depends entirely on the concentration-gradient of Na^+ and K^+ across the membrane, and that this transport system is electrogenic.

DISCUSSION

Kinetic analysis of L-glutamate and L-aspartate transport into dog erythrocytes showed no difference in K_m values for either of the amino acids between high K^+ and low K^+ cells (normal dog erythrocytes), whereas it showed a significant enhancement for V_{max} values in the high K^+ cells (Table I). This velocity effect appears to originate from the difference of Na^+ concentration-gradients between the two types of erythrocytes. Furthermore, ouabain sufficiently reduced the uptake rates of L-glutamate and L-aspartate in the high K^+ cells in a manner of velocity effect. This is in agreement with the previous reports that the inhibition of (Na^+,K^+)-ATPase with ouabain markedly reduced the velocity of L-glutamate uptake into the central nervous synaptosomes or brain slices of rat (12, 13). Schultz *et al.* (24) and Schultz and Curran (25) also reported that ouabain inhibition of the Na^+-K^+ pump located at the contraluminal side of the epithelial cells of the small intestine prevented transepithelial accumulation of sugars as well as amino acids. In the present study, ouabain did not inhibit the two amino acids' uptake by the low K^+ cells which lacked (Na^+,K^+)-ATPase (Fig. 4). This result strongly suggest that the inhibitory action of ouabain on the transport of the amino acids in the high K^+ cells is secondary to its direct inhibition of an active Na^+ extrusion system, (Na^+,K^+)-ATPase, which is directly linked to the Na^+ gradient-dependent transport system (25, 26).

Moreover, L-glutamate and L-aspartate transport were greatly increased in the low K^+ erythrocytes treated with ATP, while the transport of these amino acids in the high K^+ cells treated with ATP greatly decreased (Fig. 6). This finding supports our previous conclusion that the Na^+ and K^+ concentration gradient created by the (Na^+,K^+)-ATPase across the cell membrane ($[Na^+]_{out} > [Na^+]_{in}$, $[K^+]_{out} < [K^+]_{in}$) might stimulate L-glutamate and L-aspartate transport into the high K^+ erythrocytes.

On the other hand, the present study demonstrated that Na^+ gradient-dependent L-glutamate and L-aspartate transport may be electrogenic in both types of dog erythrocytes. This finding is identical with the electogenicity of the Na^+ L-glutamate cotransport system observed in the synaptosome from rat cerebral cortex (15), but not in agreement with that in the renal brush-border membrane vesicles from rabbit (7, 8). In addition, since the K^+ gradient have stimulated the Na^+ gradient-dependent transport of L-glutamate and L-aspartate in both of the synaptosomes (15) and renal brush-border membrane vesicles (7), it appears that in addition to generating an electrochemical gradient, high intracellular K^+ concentration may stimulate the Na^+ gradient-dependent transport of the amino acids into the high K^+ dog erythrocytes.

Furthermore, the cross-inhibition experiments for amino acid specificity of this transport system in the high K^+ and low K^+ erythrocytes showed that the L-glutamate transport system was shared by L-aspartate, but not by D-glutamate in both types of the erythrocytes. In addition, D-aspartate seems to be a substrate for this transport system. This is in agreement with the previous reports on renal brush-border membrane vesicles (8) and plasma membrane vesicles from the rat liver (10). The above anomaly in stereoselectivity of this transport system is similar to the stereoselective anomaly shown in rat hepatocytes (16, 27) and human skin fibroblasts (16, 17), suggesting that dog erythrocytes may possess a high affinity, Na^+-dependent transport system resembling the System X_{AG}^- for anionic amino acids (16, 17, 27).

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