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Na-dependent glutamate transport in high K and high glutathione (HK/HG) and high K and low glutathione (HK/LG) dog red blood cells

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Abstract

Na-dependent glutamate (Glu) influxes in the red blood cells of normal low K (LK), high K and high glutathione (HK/HG), and high K and low glutathione (HK/LG) dogs were compared. The ranges of the influxes in LK, HK/HG and HK/LG cells were 1.0–63, 62–174 and 1.3–26 μmol/l cells per h, respectively. Some LK and HK/LG dogs had red blood cells with extremely low Glu influxes. In cells with extremely low Glu influxes, however, there were clear Na-dependent Glu influxes. In LK, HK/HG and HK/LG cells, the K_m of Na-dependent Glu influx with respect to the medium Glu concentration were 17, 20 and 19 mM, respectively, and the half-maximal activation ($K_{1/2}$) with respect to medium Na concentration was 39, 40 and 42 μM, respectively. By the addition of harmaline, a hallucinogenic alkaloid, the V_{max} in LK cells was not affected and the K_m was increased, while the V_{max} was decreased and the K_m increased in HK/HG and HK/LG cells. The K_i value with harmaline by means of Dixon plot in LK cells was 5.2 mM, against 1.8 and 1.9 mM in HK/HG and HK/LG cells, respectively. These results suggest that the difference in the Na-dependent Glu influxes between 2 HK groups was due to the varying quantity, not the quality, of the transporter.

Keywords: Glutamate transport; Sodium ion dependence; Kinetics; Harmaline; Red blood cell; (HK dog)

1. Introduction

There are many amino acid transport systems in red blood cells, and the transport systems reportedly vary among different species [1,2]. These transport activities decrease with maturation of reticulocytes into mature red blood cells. Though many of these transport systems are thought to be nonfunctional relics of the reticulocyte stage, there is also another possible reason for the existence: some transporters may serve as the amino acid transporter to provide glutathione (GSH) precursors [3,4], some amino acids are an accidental substrate of other transporter such as Band 3 [4], and red blood cells have the role of an interorgan transporter of amino acid [5,6].

Dog and other carnivore red blood cells possess high affinity Na-dependent glutamate (Glu) transport, while red blood cells in most other species are essentially impermeable to Glu [7,8]. In high K dog red blood cells, in which the cellular cation composition is high K and low Na due to the existence of the Na,K-pump, and the GSH concentration is high (HK/HG cells), Na-dependent Glu transport activity is enhanced because of the greater inward Na gradient [9,10]. In HK/HG cells, the enhanced Glu influx causes a high Glu concentration, which then results in a high GSH concentration [10,11]. Na-dependent Glu transport in normal low K (LK) cells treated with ATP to change the cation composition to high K and low Na was enhanced to almost the same activity as that in HK/HG cells [9]. Therefore, it seemed that Na-dependent Glu transporter itself was not different in LK and HK/HG cells, but the difference in Na-dependent Glu transport was due to the difference in cation composition between the two cell groups, either with or without the presence of the inward Na-gradient. This difference of cation composition also affected other cation transports [12].

Recently, however, we found a dog possessing variant red blood cells with high K and low GSH (HK/LG cells)

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[13]. HK/LG cells showed low Na-dependent Glu transport activity, though the steep inward Na gradient was the same as in HK/HG cells. Thus, there must be a clear difference in the Na-dependent Glu transporter between HK/HG and HK/LG cells. Considering that the Na-dependent Glu transporter has several physiological roles, such as control of synaptic transmission [14] and absorption of the amino acid in intestine [15] and kidney [16], it is important to investigate the varying characters of the transporter among the variant cells. Here, we compared the characteristics of Na-dependent Glu transports in LK, HK/HG and HK/LG cells.

2. Materials and methods

Blood from 16 LK, 7 HK/HG and 14 HK/LG dogs was used. Blood was drawn by venipuncture into heparinized syringes and was centrifuged for less than an hour to separate red blood cells from plasma. L-[^{3}H]Glutamate (925 GBq/mmol) was purchased from Dupont. Harmaline, the hallucinogenic alkaloid, was purchased from Wako Chemical, and other chemicals used were commercial first grade.

Na-dependent Glu transport activities were measured as described elsewhere [10,13]. At first, the cells were washed in an isosmotic solution of the following composition (in mM): 150 NaCl (or *N*-methyl-D-glucamine-HCl (NMDG-Cl)), 10 Tris-HCl, 295 ± 5 mosM, pH 7.4 at 4 C. The washed cells were suspended to yield hematocrit values around 18% in an isosmotic solution of the following composition (in mM): 130 NaCl (or NMDG-Cl), 5 KCl, 15 Tris-3-(*N*-morpholino)propanesulfonic acid (Tris-Mops), 2 $MgCl_2$, 10 glucose, and 0.1% bovine serum albumin, 295 ± 5 mosM, pH 7.4, at 37 C. The flux experiment was started by addition of 150 μl of incubation solution containing an appropriate concentration of Glu with the radioactive Glu (37 kBq/ml) as a tracer, into 150 μl of cell suspension. The incubation was stopped at 5, 35 and 65 min by addition of 1 ml of ice-cold 110 mM $MgCl_2$-10 mM Tris-HCl, pH 7.4, and washed three times with the same solution.

The pellets were then lysed with 0.5 ml of 0.5% Triton X-100, and deproteinized with 5% trichloroacetic acid, followed by centrifugation at 9000 × *g* for 5 min. The radioactivity in the supernatants was measured by use of a liquid scintillation counter and a scintillant. The influx was expressed in μmol per l of cells per h. The Na-dependent influx was the difference between the influx in the media containing Na and that in the media containing NMDG.

Several kinetic parameters were measured: first, the Glu influx as function of increasing medium Na, applying the Hill plot [17] for data analysis: then, Na-dependent Glu influx as function of increasing medium Glu with K_m and V_{max} calculated by linear regression analysis of a plot of s/v against s. The effect of harmaline on the Glu influx was also examined by Dixon plot [18], and the K_i value was calculated by regression analysis.

For statistical analysis, paired *t*-test was used for the effect of inhibitor on the Glu transport activities and Student's *t*-test was used for the other experiments.

3. Results

Na-dependent Glu influxes in LK, HK/HG and HK/LG cells are shown in Fig. 1. The range of Na-dependent Glu influx in LK cells was 1 to 63 μmol/l cells per h, in HK/HG cells 62 to 174 μmol/l cells per h, and in HK/LG cells 1.3 to 26 μmol/l cells per h. The Glu influxes in 5 dogs from 16 LK dogs and 8 dogs from 14 HK/LG dogs were less than 5 μmol/l cells per h. Thus, some LK and HK/LG dogs had red blood cells with extremely low Glu influx (ELT cells). However, ELT cells were not found in HK/HG dogs.

In Fig. 2, Glu influxes in ELT cells of both LK and HK/LG cells were compared with those of red blood cells in several animals. Glu influxes in these cells were very small compared to normal LK or HK/HG dog red blood cells. In human, pig and cattle red blood cells, there were no difference between the influxes in Na and NMDG medium. Thus, these red blood cells did not show Na-dependent Glu influx. However, in the ELT cell groups, Glu influxes in Na were around 2-times higher than those in NMDG. Thus, these cells showed Na-dependent Glu influx, though the influxes were very small compared to normal LK dog red blood cells.

Glu influx as a function of medium Na is shown in Fig. 3, and the Hill coefficient and half-maximal activation for Na ($K_{1/2}$) were calculated as indicated in Table 1. At a low Na concentration from 0 to 7 mM, Glu influx in all three cell groups was sigmoidal, and at 15 mM Na, increased hyperbolically with the increasing Na concentra-

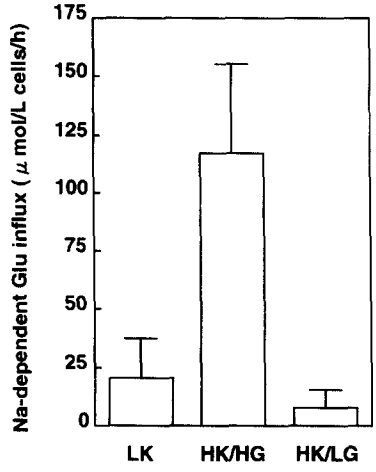

Fig. 1. Na-dependent glutamate (Glu) influxes in LK (n = 16), HK/HG (n = 7) and HK/LG (n = 14) dog red blood cells. Vertical bars indicate means + S.D.

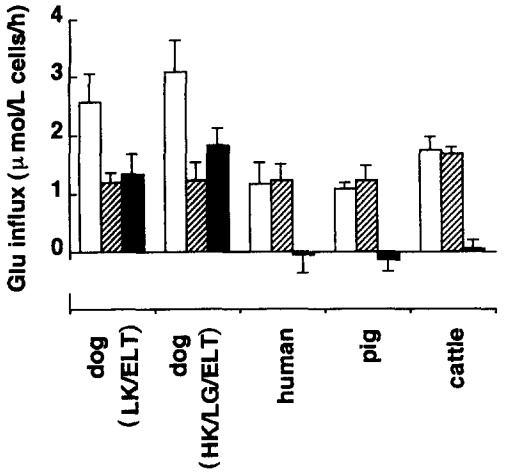

Fig. 2. Glutamate (Glu) influxes in the red blood cells from dogs (the ELT cells in the LK ($n = 4$) and HK/LG ($n = 3$)), human ($n = 4$), pig ($n = 4$), and cattle ($n = 4$). Open columns, Glu influx in the medium in which Na was used for the cation; oblique line columns, the influx in the medium in which *N*-methyl-D-glucamine (NMDG) was used for the cation; filled columns, Na-dependent Glu influx. Vertical bars indicate means + S.D.

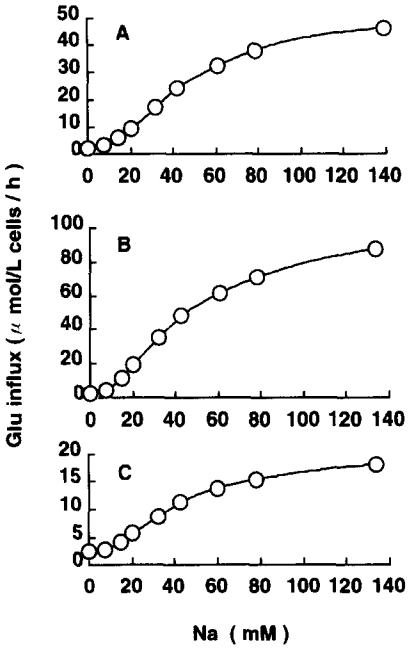

Fig. 3. Glutamate (Glu) influx as a function of increasing Na concentration in LK (A), HK/HG (B) and HK/LG (C) dog red blood cells. Na was replaced with *N*-methyl-D-glucamine to maintain osmolarity in the flux medium. Each point is the mean of two determinations. Typical experiments for three similar experiments are shown.

tion. These sigmoidal curves of activation of the Glu influxes with increasing Na were similar among the three cell groups. There were no statistical differences in the Hill coefficients and the $K_{1/2}$ values among LK, HK/HG and HK/LG cells. The Hill coefficients were 1.9 to 2.3 in the three cell groups, the cooperativity with Na was thus positive, and more than 2 Na ions were involved in the transport.

The kinetic analysis of Na-dependent Glu influx as a function of the medium Glu concentration with or without harmaline is given in Table 2. The K_m values in the three cell groups were in the same range, and there were no significant differences among the three cell groups by Student's *t*-test. Thus, among three cell groups, there were no differences in the affinity of the transporter for Glu.

Harmaline decreased the V_{max} of Na-dependent Glu influx in HK/HG and HK/LG cells by 61 and 70%, respectively. In LK cells, however, the V_{max} was not affected by harmaline. In HK/HG and HK/LG cells, the K_m was significantly increased by adding harmaline. Thus, the inhibition type was a mixed type in HK/HG and HK/LG cells. Though the K_m in LK cells was increased in each dog red blood cells, there were no significant difference between control and the cells treated with har-

Table 1
Half-maximal activation ($K_{1/2}$) and Hill coefficient of Glu influx as a function of the concentration of medium Na in LK, HK/HG and HK/LG dog red blood cells

Dogs (n)	$K_{1/2}$ (mM)	Hill coefficient
LK (3)	38.7 ± 2.9	2.09 ± 0.16
HK/HG (3)	40.3 ± 1.2	2.13 ± 0.06
HK/LG (3)	41.7 ± 3.1	2.11 ± 0.20

Values presented are means ± S.D.

Table 2
Kinetic parameters of Na-dependent Glu influxes with or without 2 mM harmaline in LK, HK/HG and HK/LG dog red blood cells

Dogs (n)		Control		2 mM harmaline	
		K_m (μM)	V_{max} (μmol/l cells per h)	K_m (μM)	V_{max} (μmol/l cells per h)
LK (3)	mean	17.3	49.3	22.0 (129) #	47.3 (98)
	S.D.	3.5	16.6	2.6 (12.7)	16.0 (2)
HK/HG (3)	mean	20.0	115	27.0 * (130)	71 * * (61)
	S.D.	2.0	26.2	2.8 (4.4)	26.5 (9.6)
HK/LG (3)	mean	19.3	21.3	31.3 * (162)	15.0 * * (70)
	S.D.	2.1	4.0	4.7 (11.7)	2.6 (3.2)

Percent of control was indicated in parentheses. In each dog, percent of control was calculated, then the mean and S.D. values were calculated.
* Significantly different at $P < 0.05$ from control by using Student's *t*-test.
* * Significantly different at $P < 0.05$ from control by using paired *t*-test.

Table 3
The K_i of Na-dependent glutamate (Glu) influx with harmaline in LK, HK/HG and HK/LG dog red blood cells

Dogs (n)	K_i (mM)
LK (3)	5.2 ± 0.9
HK/HG (3)	1.8 ± 0.3 *
HK/LG (3)	1.9 ± 0.4 *

* Significantly different at $P < 0.05$ from LK cells by using Student's t-test.
Values presented are means ± S.D.

maline. If the K_m was compared as % of control in each dog, the K_m was increased 129 ± 12.7%, suggesting competitive type inhibition. Thus, the effects of harmaline on the kinetics of Na-dependent Glu influx were no different between HK/HG and HK/LG cells, however, the effect on LK cells was different from that in the HK group cells.

Dixon plot of harmaline inhibition on Na-dependent Glu influx are listed in Table 3. There was no difference in the K_i values of the influxes between HK/HG and HK/LG cells. However, the K_i value in LK cells was 2.8-times higher than HK/HG and HK/LG cells. Thus, the inhibition was greater in HK cell groups than in LK cells.

4. Discussion

The ranges of Na-dependent Glu influxes in the three cell groups were significantly different, in particularly for influxes between HK/HG and HK/LG cells. The Na-dependent Glu influx in HK/LG cells was lower than in HK/HG cells as reported before [13]. In the report, the influxes were 20 and 30 μmol/l cells per h in two HK/LG dogs, respectively, while the influx was 110 to 200 μmol/l cells per h in HK/HG cells.

In the present study, the red blood cells from 57% of HK/LG and 31% of LK dogs were ELT cells. Some dogs thus possess red blood cells with extremely low Na-dependent Glu transport activity. Though extremely low, Na-dependent Glu influxes were confirmed in the ELT cells of both LK and HK/LG cells. Thus, the influxes in the ELT cells differed from those in the other animals with red blood cells showing no Na-dependent Glu influx (Fig. 2). Such low Na-dependent Glu influx was not reported before in dog red blood cells suggesting existence of a variant with a Na-dependent Glu transporter defect in both the HK and LK dog groups. The wide range of the activities of Na-dependent Glu influxes and the existence of ELT cells suggest that the gene or the expression process of the Na-dependent Glu transport is not simple.

The difference in the Na-dependent Glu influxes between HK/HG and HK/LG cells appears to be quantitative regarding the transporter rather than qualitative, for the following reasons. The K_m value of Na-dependent Glu influx was the same in these two cell groups. Furthermore, the changes of K_m and V_{max} by the addition of harmaline were the same in HK/HG and HK/LG cells. The results of the Na-dependency on the Glu influx also support this assumption. The values of Hill coefficients indicated that the conformational change of the transporter by binding with Na was the same among the three cell groups (Fig. 3 and Table 1). Thus, these results indicated that the transporters in HK/HG and HK/LG cells were qualitatively identical whereas transporters in HK/HG cells may outnumber those in HK/LG cells.

Harmaline is known to inhibit several Na-dependent transporters, as well as the Na dependent Glu influx in kidney brush border [19] and muscle fiber [20]. Harmaline was also reported as a Na-site inhibitor in the Na-dependent amino acid transporter [21,22]. The inhibition by addition with harmaline of Na-dependent Glu influx in HK/HG and HK/LG cells was of mixed type, indicating that the binding of harmaline to the transporter affected the affinity for Na or Glu to the transporter. However, the inhibition of the Na-dependent Glu influx in LK cells was of competitive type, i.e., binding of harmaline to the transporter competed with Na or Glu. In LK cells, the cellular cation composition is different from HK cells, and the cis-trans Na-gradient in LK cells is different from HK cells. Hence the kinetic constants in LK cells cannot be simply compared with that in the HK group cells. The effect of harmaline on the transporter may be modified by a change in the Na gradient, because Na binding to the transporter may be different between cells with opposite Na gradient.

As shown in Fig. 3, the activation of the influx by increasing the medium Na concentration was shown for very low Na concentrations in LK cells. Thus, for the Na-dependent Glu influx, the inward Na gradient appears to be dispensable, but not the presence of Na in the medium. This result corresponds to the stoichiometry of ion coupling in Na-dependent Glu transport in the salamander retinal glia [23]. In the glial cells, the driving force of Glu transport was supplied by a chemical gradient with not only medium Na but also intracellular K and OH or HCO_3. In dog red blood cells, Na-dependent Glu transport activity was enhanced by intracellular K and HCO_3 [24] same as in the glial cells.

Though the implications of the variation of the Na-dependent Glu transport activity can not be explained at present, the changes of the Glu influxes among dog red blood cells examined in this study were useful to understand the role of the transport. Further comparative studies are necessary to understand more fully the significance of the variation in Glu transport.

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