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Characterization of Na^+-dependent L-glutamate transport in canine erythrocytes

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Abstract

Characteristics of the high-affinity Na^+-dependent L-glutamate transport system in canine erythrocytes were studied by using intact cells and resealed ghosts. The L-glutamate transport showed a precise dependence on extracellular Na^+ and intracellular K^+. Kinetical analysis revealed that two Na^+ ions and one K^+ ion were involved in each L-glutamate transport cycle. The L-glutamate transport was inhibited most potently by *threo*-3-hydroxyaspartate and L-cysteinesulfinate (at 25 μM, 83% and 79% inhibition, respectively) and weakly by dihydrokainate and DL-α-aminoadipate (at 25 μM, 21% and 17% inhibition, respectively). From these stoichiometrical and pharmacological properties we concluded that the L-glutamate transport system in canine erythrocytes is a product of the L-glutamate transporter gene family and resembles a neuronal transporter rather than a glial one. L-Glutamate uptake was increased by internal, but not external, HCO_3^- when the internal and external anions of the erythrocytes were replaced by several other anions. Moreover, this enhancement was blocked by inhibition of carbonic anhydrase, which indicated that L-glutamate transport was at least partly dependent on HCO_3^- generated inside the cells. These observations indicate that anion countertransport is coupled to the high-affinity Na^+- and K^+-dependent L-glutamate transport in canine erythrocytes.

Keywords: Glutamate transport; Ion dependence; Anion countertransport; Erythrocyte; (Dog)

1. Introduction

A high-affinity Na^+-dependent L-glutamate and L-aspartate transport system, designated system X_{AG}^-, has been found in a variety of tissues and cells [1–8]. The transporter plays important physiological roles such as terminating neurotransmission in the central nervous system [9] and absorbing these amino acids in the kidney [2] and small intestine [3]. Recently, several different Na^+-dependent L-glutamate transporters were cloned in rat brain [10,11] and rabbit small intestine [12]. Although they are very similar in primary structure, their tissue localizations are quite different from each other [10–12]. Thus it is believed that Na^+-dependent L-glutamate transporters are products of a gene family including various isoforms.

Canine erythrocytes possess an Na^+-dependent electrogenic transport system for L-glutamate and L-aspartate [13] in contrast to most mammalian erythrocytes, which are impermeable to these amino acids [14]. Erythrocytes with high concentrations of intracellular K^+ (HK cells) incorporate these amino acids more actively than LK cells because of the increased Na^+ gradient across the plasma membrane, resulting in accumulation of L-glutamate, and, consequently, in an increased concentration of glutathione [15]. Therefore, in canine erythrocytes, the transporter serves to supply L-glutamate as the substrate for production of glutathione to protect the cells from oxidative stress [15].

These transporters showed similar characteristics in their Na^+ dependence, affinities to the substrates and electrogenicity [13]. However, the stoichiometry of ion coupling, that is, what kind of and how many ions are involved in the electrogenic pathway, has not been precisely determined [16]. Recently, Bouvier et al. [17] demonstrated a unique complicated stoichiometry of L-glutamate transport in salamander retinal glial cells that was coupled to OH^- (or HCO_3^-) countertransport in addition to cotransport of two Na^+ ions and countertransport of one K^+ ion. The

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purpose of the present study was to evaluate if such complicated stoichiometry exists in the mammalian L-glutamate transport system. In the present study, we examined the ionic dependence on cations (Na^+ and K^+) and various anions, and substrate specificities of the canine erythrocyte L-glutamate transport system. The results demonstrated that the transport system in canine erythrocytes was stoichiometrically and pharmacologically similar to that in brain neuronal cells [12] and involved the countertransport of anions.

2. Materials and methods

2.1. Chemicals

The sources of chemicals were as follows. L-[3,4-^{3}H] Glutamic acid (49 Ci/mmol) was from Du Pont/New England Nuclear (Boston, MA, USA). L-Glutamic acid, D-glutamic acid, L-aspartic acid, D-aspartic acid and acetazolamide from Wako Pure Chemical Industries (Osaka, Japan), *threo*-3-hydroxyaspartate from Tocris Neuramine (Bristol, UK), dihydrokainate (2-carboxy-4-isopropyl-3-pyrrolidine acetic acid, DHK), DL-α-aminoadipic acid, L-cysteinesulfinic acid, phenylmethylsulfonyl fluoride (PMSF) and valinomycin from Sigma (St. Louis, MO, USA), and 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS) from Dojin (Kumamoto, Japan). All other chemicals were of the purest grade available.

2.2. Preparation of erythrocyte suspension

Preparation of erythrocyte suspension was carried out as previously described with some modification [13]. Heparinized venous blood from HK or LK dogs was washed three times at 4°C with an incubation medium containing 150 mM NaCl, 1 mM $MgCl_2$, 5 mM D-glucose, 0.1% (w/v) bovine serum albumin (BSA) and 10 mM 3-(*N*-morpholino)propanesulfonic acid (Mops)/Tris (pH 7.4). When Na^+ dependence and substrate specificities were examined, the incubation medium also contained 5 mM KCl. The washed cells were resuspended in the same medium to yield a hematocrit value of 20%.

2.3. Preparation of the resealed ghosts

Resealed ghosts were prepared according to the procedure of Klonk and Deuticke [18]. Heparinized venous blood from LK dogs was centrifuged at $1000 \times g$ for 5 min and plasma and buffy coats were removed by aspiration. The cells were washed three times with Na^+-and K^+-free phosphate buffer (150 mM choline chloride, 1 mM $MgCl_2$ and 10 mM orthophosphoric acid/Tris (pH 7.4)) at 4°C. The last centrifugation was done at $2000 \times g$ for 10 min and supernatant was sufficiently removed. One volume of packed cells was lysed in 40 volumes of the hypotonic buffer (5 mM orthophosphoric acid/Tris (pH 7.4), 1 mM $MgCl_2$, 0.8 mM PMSF) with stirring for 10 min on ice. Unsealed ghosts were pelleted by centrifugation at $15\,000 \times g$ for 20 min. One volume of unsealed ghosts was diluted with an equal volume of hypotonic buffer followed by the addition of 0.5 volume of 5 × reconstitution buffer containing 750 mM KCl or choline chloride, 100 mM Mops/Tris (pH 7.4) with gentle stirring on ice for 10 min. The internal K^+ concentration was changed by a combination of the reconstitution buffers (see figure legends for details). This mixture was incubated for 45 min at 37°C and centrifuged for 5 min at $15\,000 \times g$. The supernatants were thoroughly removed and resealed ghosts were obtained as pink pellets.

2.4. Measurement of L-glutamate transport

L-Glutamate uptake was measured as previously described [13]. Briefly, 100 μl of the incubation medium containing 1–5 μCi/ml L-[^{3}H]glutamate was added to 100 μl of the erythrocyte suspension and incubated at 37°C for the indicated periods. Structural analogues of L-glutamate were added at 25 μM, when present. Incubation was stopped by the addition of 1 ml of ice-cold incubation medium and the cells were washed with the same buffer by repeated centrifugation ($15\,000 \times g$, 10 s). After the final wash, cells were lysed by the addition of 200 μl of 0.5% (v/v) Triton X-100 followed by the addition of 200 μl of 5% (w/v) trichloroacetic acid and centrifuged ($15\,000 \times g$, 3 min). The resulting 200-μl supernatants were counted for radioactivity using the solid scintillant Ready Cap (Beckman, Fullerton, CA, USA). The results were expressed as nmol/ml cells.

When the internal anions were replaced, HK cell suspension were prepared as described above. The cells were washed three times with medium containing 150 mM NaX (X = Cl, F, Br, I) or 25 mM $NaHCO_3$/5% CO_2 + 125 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 5 mM D-glucose, 0.1% BSA and 10 mM Mops/Tris (pH 7.4), at 4°C and placed at room temperature (22°C) for 5 min to load the cells with each anion via an anion exchanger (band 3) [19]. 50 μl of the supernatant were removed from the cell suspension after rapid centrifugation ($15\,000 \times g$, 10 s) and resuspended cells were preincubated at 37°C for 1 min. Uptake of L-glutamate was measured at 37°C for 1 min by the addition of 250 μl of medium containing 150 mM Na gluconate, 5 mM KCl, 1 mM $MgCl_2$, 5 mM D-glucose, 0.1% BSA, 10 mM Mops/Tris (pH 7.4), 6 μM L-[^{3}H]glutamate, 6 μM valinomycin and 12 μM DIDS. Movement of anions across the membrane was diminished by DIDS, which is the specific inhibitor for band 3 [19]. Valinomycin was also added to abolish the effect of the membrane potential, which is altered when anion exchange is inhibited [20]. Thus, 125 mM Na gluconate, 25 mM Na salts of loaded anions, 5 μM L-[^{3}H]glutamate and valinomycin, and 10 μM DIDS were present outside the cells in

this system. To assess the effect of external anion substitution, the cells were suspended in the medium containing 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 5 mM D-glucose, 0.1% BSA, and 10 mM Mops/Tris (pH 7.4), with an Ht value of 20%. The L-glutamate uptake was measured at 37°C for 1 min in medium containing 150 mM NaX (X = Cl, F, Br, I) or 25 mM $NaHCO_3$/5%CO_2 + 125 mM NaCl using the same procedure as for the internal anion substitution.

When L-glutamate uptake in resealed ghosts was measured, 190 μl of medium containing 150 mM NaCl, 1 mM $MgCl_2$, 20 mM Mops/Tris (pH 7.4), and 1–5 μCi/ml L-[^{3}H]glutamate was added to 10 μl of resealed ghosts. After the incubation, the resealed ghosts were washed with the same medium three times by centrifugation at 15 000 × *g* for 30 s. 100 μl each of 0.5% Triton X-100 and 5% trichloroacetic acid were added and centrifuged and the resulting supernatant was used for counting radioactivity. The results were expressed as nmol/mg protein. The protein contents of resealed ghosts were determined by the method of Bradford [21] with bovine serum albumin as the standard. To determine the membrane protein contents of the resealed ghosts, hemoglobin-depleted ghosts were prepared from the resealed ones by hypotonic lysis and washing [22].

The efflux of L-glutamate was measured in the LK cells preloaded with 40 nM (2 μCi/ml) L-[^{3}H]glutamate at 37°C for 30 min in medium containing 150 mM NaCl, 1 mM $MgCl_2$, 5 mM D-glucose, 0.1% BSA, and 10 mM Mops/Tris (pH 7.4). The loaded cells were washed three times with the ice cold efflux medium containing NaCl and/or KCl (NaCl + KCl = 150 mM), 1 mM $MgCl_2$, 5 mM D-glucose, 0.1% BSA, 10 mM Mops/Tris (pH 7.4). 50 μl of the cell suspension (Ht = 20%) were added to 1 ml of the same efflux medium. After incubation for the indicated period at 37°C, the radioactivity of L-[^{3}H]glutamate retained in the cells was determined as described above. The efflux was expressed as a percentage of the retained radioactivity at time 0.

3. Results

The ionic dependence of L-glutamate transport in canine erythrocytes was investigated in intact cells and resealed ghosts. The dependence on external Na^+ was determined in LK cells to minimize the association of the K^+ gradient (Fig. 1). The sigmoidal dependence on the Na^+ concentration suggested that multiple Na^+ ions were involved in the transport process. Analysis by Hill's equation showed a Hill coefficient of 2.01 and half activation at 71 mM [Na^+]out (Fig. 1, inset), indicating that two Na^+ ions were involved in the transport process.

Fig. 2 shows that high-affinity Na^+-dependent L-glutamate transport was reconstituted in the resealed ghosts that contained K^+ as the internal cation. However, when

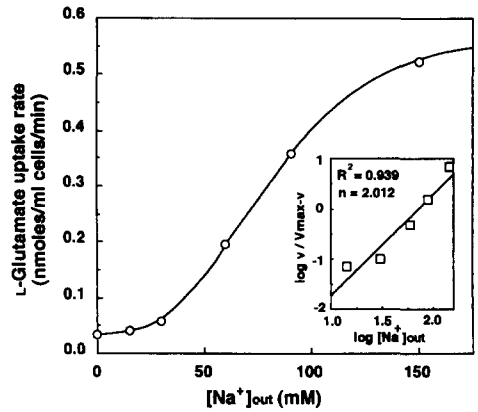

Fig. 1. The dependence on the external Na^+ of L-glutamate transport in canine erythrocytes. Uptake of 5 μM L-[^{3}H]glutamate (1 μCi/ml) was measured at 37°C for 2 min in medium containing *x* mM NaCl (*x* = 0–150) and 150 − *x* mM choline chloride. Data are expressed as mean values of experiments in duplicate. Linear transformation of data expressed as the Hill's equation is shown in the inset.

internal K^+ was replaced by choline, L-glutamate uptake was totally abolished (Fig. 2). Thus, it was demonstrated that not only external Na^+ but also internal K^+ was required for the L-glutamate transport, as demonstrated for the transporter in rat brain [1,10]. We surveyed the dependence of the uptake on the internal K^+ concentration and K^+ gradient in resealed ghosts (Fig. 3). External K^+ inhibited L-glutamate transport according to its concentration, suggesting that the outward gradient of K^+ facilitated the uptake. This is consistent with the model in which the efflux of K^+ was coupled to L-glutamate influx. Kinetic analysis showed that the dependence on internal K^+ fit to a simple Michaelis-Menten equation, equivalent to Hill's model with a Hill coefficient of 1.0, with a K_m value of 23 mM and a V_{max} of 0.41 nmol/mg protein per 10 min under conditions in which $[Na^+]_{out}$ = 100 mM, $[K^+]_{out}$ = 0 and [L-glutamate]$_{out}$ = 5 μM (Figs. 3A and 3B). The first-

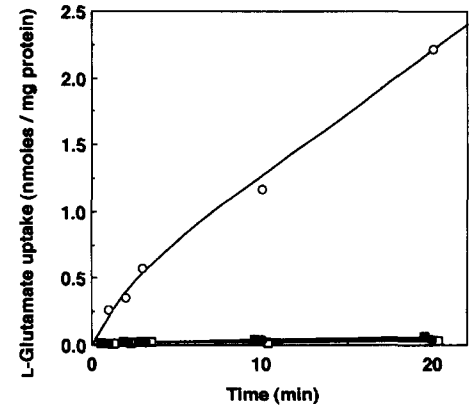

Fig. 2. The requirement of Na^+ and K^+ in L-glutamate transport. Resealed ghosts were loaded with 150 mM KCl (○,●) or 150 mM choline chloride (□,■). Uptake of 5 μM L-[^{3}H]glutamate (2 μCi/ml) was measured at 37°C for the indicated periods in medium containing 150 mM NaCl (○,□) or choline chloride (●,■). Data are expressed as mean values of experiments in duplicate.

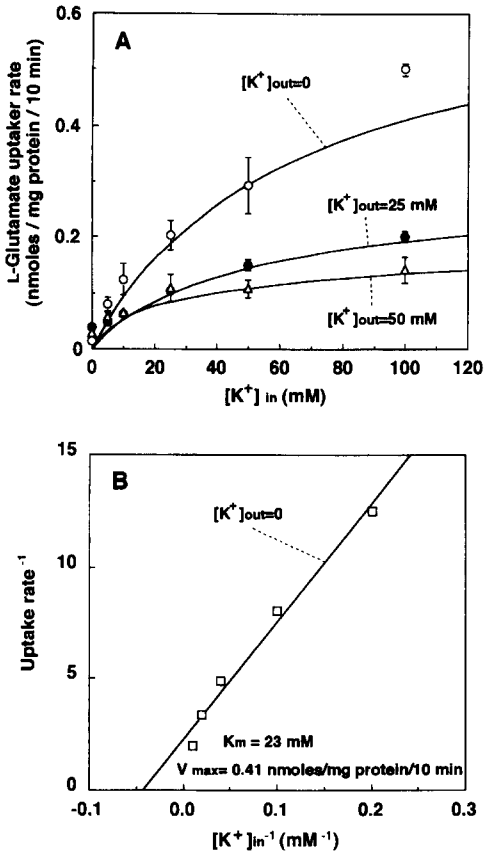

Fig. 3. The dependence on the internal K^+ of L-glutamate transport in resealed ghosts. Resealed ghosts were loaded with x mM KCl ($x = 0$–100) and $150 - x$ mM choline chloride. Uptake of 5 μM L-[^{3}H]glutamate (2 μCi/ml) (A) was measured at 37°C for 10 min in medium containing 100 mM NaCl, 0 (○) or 25 mM (●) or 50 mM (△) KCl (50 mM, 25 mM and 0 mM choline chloride were added, respectively). Data are expressed as means ± S.D. of experiments in triplicate and linearized by Lineweaver-Burk plot (B) with the data of $[K^+]_{out} = 0$.

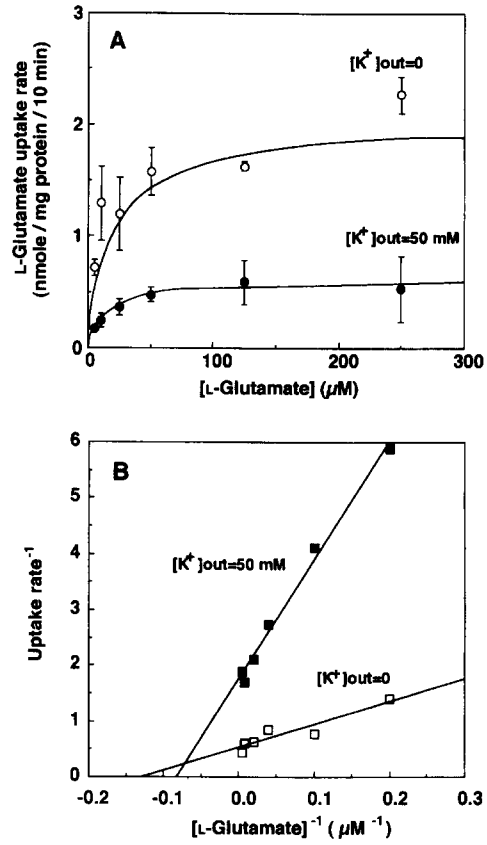

Fig. 4. Effect of the external K^+ and L-glutamate concentration on the uptake of L-glutamate in resealed ghosts. Resealed ghosts were loaded with 100 mM KCl, 50 mM choline chloride. Uptake of L-[^{3}H]glutamate (1–5 μCi/ml) by the resealed ghosts (A) was measured at 37°C for 10 min in medium containing 100 mM NaCl, 50 mM KCl (●) or choline chloride (○) and various concentration of L-glutamate. Data are expressed as means ± S.D. of determinations in triplicate and linearized by Lineweaver-Burk plot (B).

order dependence of glutamate uptake suggested that one K^+ ion was involved in the transport process.

The dependence on the external L-glutamate concentration was also described by a Michaelis-Menten equation with a K_m value of 7.2 μM and a V_{max} of 1.89 nmol/mg protein per 10 min ($[Na^+]_{out} = 100$ mM, $[K^+]_{out} = 0$). This first-order dependence suggested that one L-glutamate was associated with the transport cycle (Fig. 4). When the external K^+ concentration was raised to 50 mM ($[Na^+]_{out} = 100$ mM), the V_{max} value decreased to 0.57 nmol/mg protein per 10 min without a significant change of the K_m value (12.1 μM), indicating that the inhibition by external K^+ (Figs. 3 and 4) was not caused by a change of affinity to L-glutamate.

Fig. 5 shows the efflux of L-glutamate from canine erythrocytes. Approx. 90% of L-glutamate within the cells was retained even after 2 h incubation when the inward Na^+ and outward K^+ gradients were present ($[Na^+]_{out} = 150$ mM and $[K^+]_{out} = 0$). However, retained L-glutamate was markedly decreased when the extracellular concentrations of Na^+ and K^+ were changed to 100 mM or less and

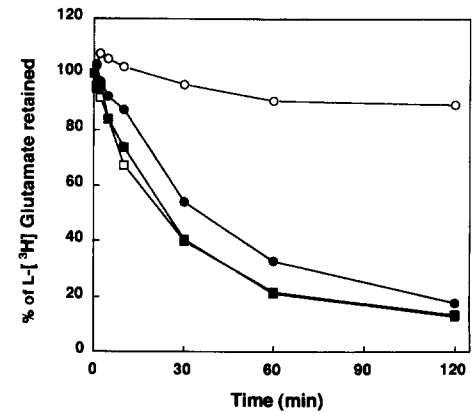

Fig. 5. The efflux of L-glutamate from canine erythrocytes. LK cells were loaded with 40 nM L-[^{3}H]glutamate (2 μCi/ml) in medium containing 150 mM NaCl at 37°C for 30 min. Percentages of the retained L-[^{3}H]glutamate in the cells were measured after incubation of the L-[^{3}H]glutamate loaded cells for the indicated periods at 37°C in medium containing 150 mM NaCl (○), 100 mM NaCl + 50 mM KCl (●), 50 mM NaCl + 100 mM KCl (□) and 150 mM KCl (■). Data are expressed as mean values of determinations in duplicate.

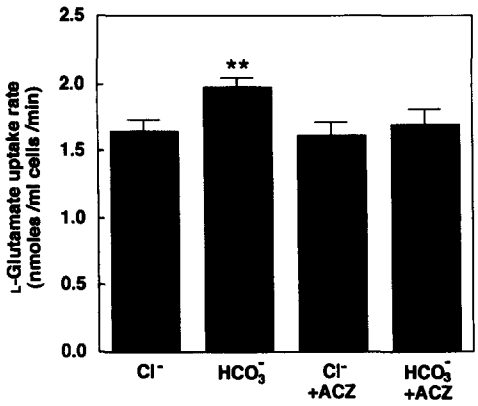

Fig. 6. Effect of HCO_3^-/CO_2 buffer on L-glutamate transport in canine erythrocytes. Uptake of 5 μM L-[^{3}H]glutamate (1 μCi/ml) was measured at 37°C for 1 min in medium containing 150 mM NaCl or 135 mM NaCl + 15 mM $NaHCO_3$ /5%CO_2 with or without 1 mM acetazolamide (ACZ). Data are expressed as means ± S.D. ($n = 7$, $^{**}P < 0.01$, t-test compared with the uptake value in Cl^- without ACZ).

50 mM or more, respectively, which were sufficient to reverse the gradient directions of these cations in LK cells [15]. These results suggested that L-glutamate can be transported by the transporter in the opposite direction using the reverse gradients of Na^+ and K^+ as the driving forces. This could be the cause of the apparent inhibition of the transport by external K^+ described above.

These results showed that Na^+ and K^+ are completely coupled to L-glutamate transport, and that two Na^+ and a K^+ are involved in each transport cycle. This stoichiometry is electroneutral while the transport process is considered to be electrogenic in intact cells [13] and resealed ghosts (data not shown). Therefore, we examined the effect of replacement of intracellular or extracellular Cl^- by several other anions. Extracellular HCO_3^- (added as 15 mM $NaHCO_3$/5% CO_2) increased the uptake by 20% as shown in Fig. 6. This effect of extracellular HCO_3^- disappeared in the presence of acetazolamide, a carbonic anhydrase inhibitor, although acetazolamide itself did not change the uptake in the Cl^- medium (Fig. 6). When the uptake was measured in the presence of DIDS and valinomycin, some halides increased the L-glutamate uptake in the order $F^- > Br^- > Cl^- \geq I^-$ despite their intra- and extracellular distribution (Fig. 7). Under the same condition, the cells loaded with 25 mM HCO_3^- showed an increase in transport of L-glutamate by 55% that was as intensive as in the F^--loaded cells, whereas the addition of 25 mM HCO_3^- outside the cells did not affect the uptake. These results demonstrated that HCO_3^- accumulated inside the erythrocytes facilitated L-glutamate transport.

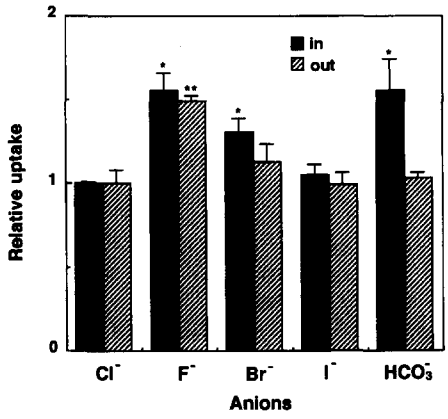

Fig. 7. Effect of diverse anions on L-glutamate transport in canine erythrocytes. HK erythrocytes were loaded with diverse anions (■, in) by incubation at 22°C for 5 min in medium containing 150 mM NaX (X = Cl, F, Br, I) or 125 mM NaCl + 25 mM $NaHCO_3$ /5%CO_2. The uptake of 5 μM L-[^{3}H]glutamate (1 μCi/ml) was measured at 37°C for 1 min in medium containing 150 mM Na gluconate in the presence of 10 μM DIDS and 5 μM valinomycin. In order to replace the external anion (out), uptake of 5 μM L-glutamate were measured with HK erythrocytes loaded with Cl^- in medium containing 150 mM NaX (X = Cl, F, Br, I) or 125 mM NaCl + 25 mM $NaHCO_3$ /5%CO_2 at 37°C for 1 min in the presence of 10 μM DIDS and 5 μM valinomycin. Data are expressed as means ± S.D. of the relative uptake ($Cl^- = 1.0$, $n = 3$, $^*P < 0.05$, $^{**}P < 0.01$, t-test compared with the uptake value in Cl^-).

Table 1
Cross-inhibition of L-glutamate transport in dog erythrocytes by structural analogues

Inhibitors	L-Glutamate uptake (% of control)
None	100.0 ± 5.0
L-Glutamate	22.3 ± 0.3
D-Glutamate	76.7 ± 11.8
L-Aspartate	20.3 ± 0.9
D-Aspartate	53.2 ± 2.2
threo-3-Hydroxyaspartate	16.6 ± 0.4
Dihydrokainate	78.9 ± 0.3
Dihydrokainate (1 mM)	56.4 ± 11.7
DL-α-Aminoadipate	82.8 ± 4.4
L-Cysteinesulfinate	20.6 ± 1.0

The uptake of 5 μM L-Glu was measured with HK cells at 37°C for 5 min in the media containing 25 μM of each analogue. Data are expressed as means ± S.D. of determinations in triplicate.

Cross inhibition analysis revealed that the canine erythrocyte L-glutamate transport system was highly sensitive to *threo*-3-hydroxyaspartate and L-cysteinesulfinate as well as to L-aspartate (Table 1). *threo*-3-Hydroxyaspartate is a potent inhibitor of the transporter in rat brain [11] and L-cysteinesulfinate is another candidate for physiological substrate [23]. In contrast, the use of dihydrokainate and DL-α-aminoadipate, both of which suppressed glial uptake by 70–90% [10], resulted in no obvious inhibition in the erythrocytes. Dihydrokainate appeared to be required at > 1 mM for 50% inhibition. D-Aspartate possessed a moderate inhibitory effect in agreement with our previous study [13].

4. Discussion

We demonstrated that high-affinity L-glutamate transport in canine erythrocytes is coupled to cotransport of two

Na ions and countertransport of one K ion (Figs. 1 and 3). Since the transport process is electrogenic as we reported previously [13], the stoichiometry should require additional movement of other cation(s) such as H^+ or anion(s). Bouvier et al. [17] have proposed, based on several electrophysiological investigations in salamander retinal glial cells [24], the stoichiometry involving cotransport of two Na^+ and countertransport of one K^+ and one pH changing anion for one glutamate. According to the anion involved in the transport of L-glutamate, they suggested that OH^- is the main anion transported and that occasionally HCO_3^- is transported in vivo because they did not observe a significant decrease of pH due to L-glutamate movement across the membrane of the cells in which carbonic anhydrase was blocked [17]. Our direct measurement of L-glutamate uptake demonstrated that the transport was enhanced by HCO_3^- at physiological concentrations (15–25 mM $NaHCO_3$/5% CO_2). This increase was diminished by acetazolamide, a potent inhibitor of carbonic anhydrase, suggesting a stimulatory effect of HCO_3^- accumulated within the cells (Fig. 6). The effect of HCO_3^- was more evident when the major transport system for HCO_3^- in erythrocytes, band 3 [19], was blocked with DIDS after partial substitution of HCO_3^- for internal anions (Fig. 7). These results indicated that L-glutamate transport in canine erythrocytes was stimulated in the presence of internal HCO_3^- as the substrate anion. However, L-glutamate uptake was not disturbed by HCO_3^- deprivation in resealed ghosts (Figs. 3 and 4) or in erythrocytes suspended in medium degassed and equilibrated with N_2 (data not shown), indicating that a major basal fraction of the L-glutamate uptake was linked to movement of anions other than HCO_3^-, such as OH^-. It is most likely, therefore, that glutamate transporter in canine erythrocytes has the stoichiometry in which the transport of one glutamate is accompanied by two Na^+ and by the countertransport of one K^+ and one anion (OH^- or HCO_3^-) as in salamander glial cells [17]. Although F^- and Br^- increased the L-glutamate uptake, it is unlikely that these halides, including Cl^- and I^-, were coupled to the transport, since both the external and internal replacements exhibited the same effect to a similar extent (Fig. 7).

The canine erythrocyte L-glutamate transport system also showed substrate specificity that was similar to those of transporters recently cloned (Table 1) [10–12]. The erythrocyte transport system, however, showed a rather low sensitivity to dihydrokainate and DL-α-aminoadipate, which potently inhibited a glial transporter [10] but not a neuronal one [12]. The neuronal transporter, EAAC1, was originally cloned in rabbit intestine and was shown to be distributed to many tissues including brain, intestine, kidney, liver, and heart [12]. Based on these observations, we suppose that the canine erythrocyte L-glutamate transport system is a product of the L-glutamate transporter gene family and that it is ubiquitious and neuronal. Therefore, possible regulation of the transport by HCO_3^- would occur in a variety of tissues depending on their metabolic state, including CO_2 production and its conversion to HCO_3^-. In particular, in tissues and cells exposed to relatively high levels of O_2 and CO_2, enhancement of L-glutamate uptake by HCO_3^- may be helpful to protect the contents from oxidative damage in response to O_2 consumption and CO_2 production. In fact, the increase of intracellular L-glutamate stimulates glutathione synthesis in endothelial cells [24] and canine erythrocytes [15] by releasing the feedback inhibition of γ-glutamylcysteine synthetase by glutathione [15,25,26].

In normal (LK) canine erythrocytes, L-glutamate is not accumulated as in HK cells because the intracellular Na^+ and K^+ concentrations are reversed to collapse the inward Na^+ and outward K^+ gradients due to complete loss of Na^+, K^+-ATPase during reticulocyte maturation [22]. In this stage, the L-glutamate transport system will excrete excess L-glutamate and L-aspartate since transport in the reverse direction seems to occur (Fig. 5). Thus, the Na^+ and K^+-dependent L-glutamate transport system in canine erythrocytes plays a role in regulation of these amino acids and glutathione contents at the maturational stage from reticulocyte to erythrocyte. Moreover, in other tissues and cells, the dependence of L-glutamate transport on Na^+, K^+ and HCO_3^- may be important because such ionic environments are closely related to energy metabolism and respiration.

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