Rheogenic Property of Na+/Acidic Amino Acid Co-transport by Guinea Pig Intestine

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Abstract The influx of L-glutamate or L-aspartate across the intestinal brush border membrane was absolutely dependent on the presence of Na⁺ in the mucosal solution and saturable. The addition of these amino acids into the mucosal solution induced a sudden and sustained increase in the transepithelial potential difference (PDᵣ), which was found to be absolutely dependent on the presence of Na⁺ in the mucosal solution. The increases in PDᵣ induced by L-glutamate or L-aspartate conformed to Michaelis-Menten kinetics against the concentration of each organic substrate. On the other hand, the relationship between the change in short-circuit current induced by acidic amino acid and Na⁺ concentration did not conform to Michaelis-Menten kinetics but was sigmoid. A kinetic study indicated that 2 Na⁺ were translocated per molecule of the acidic amino acid. The PDᵣ was not altered by the total replacement of K⁺ by Na⁺ in the mucosal solution and showed the optimum pH at around 7. The transfer of L-glutamate from mucosal to serosal solution was examined with everted sac of ileum. A significant increase in serosal appearance was observed for L-glutamate and L-alanine after the incubation with L-glutamate. The serosal appearance of L-glutamate was unaffected by the presence or absence of HCO₃⁻.

Key Words: intestinal absorption, active transport, co-transport, transport kinetics, acidic amino acid.

Characteristics of transmembrane transport of acidic amino acids have been studied by many authors in the proximal renal tubule (HOSHI et al., 1976a; SAMARZIA and FRÖMTER, 1982b) and the small intestine (SCHULTZ et al., 1970; BAMFORD and STEWART, 1973; LERNER and STEINKE, 1977). Most of these studies demonstrated that L-glutamate and L-aspartate were transported by a common carrier specific for acidic amino acids, and that the transport was strongly dependent on the presence of Na⁺ in the bathing medium. Similar Na⁺ dependent transport was demonstrated in brush border membrane vesicles from the small intestine (CORCELLI et al., 1982; CORCELLI and STORELLI, 1983) and kidney.
One still controversial problem is the stoichiometric relationship between Na⁺ and acidic amino acid or the involvement of other cations. SCHULTZ et al. (1970) showed that the carrier-mediated influx of acidic amino acid was strongly dependent on Na⁺ in rabbit ileum, but that a small but significant influx was still observable in the absence of Na⁺. However, such a small influx might be a diffusional entry because they also reported that the coupling ratio between Na⁺ and L-glutamate averaged 1.1 ± 0.4, which would indicate 1:1 stoichiometry of transport. On the other hand, HOSHI et al. (1976a) demonstrated that, in the proximal tubule of Triturus, the cellular uptake of L-aspartate was completely dependent on Na⁺ and the luminal membrane transport was non-electrogenic. In contrast, SAMARZIJA and FRÖMPER (1982b) showed that, in proximal tubule of the rat kidney, the luminal membrane transport of acidic amino acid was electrogenic. They suggested that the acidic amino acid was cotransported with excess Na⁺, probably in 2:1 coupling. Similar discrepant findings on the stoichiometric relationship have been shown also in brush border membrane vesicles (BURCKHARDT et al., 1980; SCHNEIDER et al., 1980; CORCELLI et al., 1982; NELSON et al., 1983).

The present study aimed at detailed analysis of coupling of Na⁺ and acidic amino acid fluxes in the guinea pig small intestine. Our preliminary observation revealed that the addition of acidic amino acid into mucosal incubation medium caused an immediate and sustained increment of transmural potential difference (PD), suggesting the presence of electrogenic mechanism. The relationships between the electrical change and Na⁺ concentration or amino acid concentration were analyzed kinetically.

METHODS

Preparation of everted intestine and influx measurement. The influxes of L-glutamate or L-aspartate into the everted ileum of guinea pig were determined as described in detail elsewhere (HIMUKAI and HOSHI, 1980; HIMUKAI et al., 1983). Briefly, the everted sac preparations were preincubated in a standard buffer solution (composition described below) for 30 min at 37°C. When the ionic composition of a test incubation medium was different from that of the standard solution, the samples were preincubated for an additional 1 min in a solution which had the same ionic composition as that of a test incubation medium. The preparations were incubated for 1 min at 37°C in buffer solutions containing an amino acid at various concentrations. The standard buffer solution employed in the present study had the following composition (mM): Na₂SO₄, 50; D-mannitol, 160; K₂SO₄, 1.25; KH₂PO₄, 0.25; CaSO₄, 1.5; MgSO₄, 1.0; Tris/H₂SO₄, 20 (pH 7.4), unless otherwise noticed. The reason for the use of SO₄²⁻-medium was also described previously (HIMUKAI and HOSHI, 1980). L-[U-¹⁴C]glutamate and L-[U-¹⁴C]aspartate were used...
as the tracers (0.2 $\mu$Ci/ml). D-[1-3H]mannitol (2 $\mu$Ci/ml) was used in order to correct for extracellular fluid adhering to the mucosal surface. Following the incubation, the preparations were rinsed with ice-cold isotonic mannitol solution, blotted, and extracted in 2 ml of 3% trichloroacetic acid. The radioactivities of the extraction fluid and the test medium were counted in a liquid scintillation counter. All data of cellular uptake rate were presented as means±S.E.M. (nmol min$^{-1}$ cm$^{-2}$ of serosal surface area).

Recording of amino acid-induced increase in PD$_t$ and short-circuit current ($I_{sc}$). These procedures were also described in detail previously (HIMUKAI et al., 1983). Recording of PD$_t$ from the everted preparations were carried out under the fixed ionic conditions using the standard buffer solution as described above. The everted ileum was fixed over a perforated polyethylene tube and incubated in 20 ml mucosal bathing solution bubbled with pure oxygen at 37°C. The PD$_t$ was recorded by means of 1 M KCl-agar bridges connected to half calomel electrodes. Even in the experiment using K$^+$ free buffer solution, 1 M KCl-agar bridges were adopted because it took only a few minutes to carry out the experiment in such a condition. After the experiment, the concentration of K$^+$ in the mucosal bathing medium was negligibly increased (examined using a flame photometer). A small amount of a high concentration amino acid solution (adjusted to pH 7 with Tris) was added to the mucosal bathing solution to give a desired final concentration.

For the purpose of measuring $I_{sc}$, the isolated ileal segments were opened along the mesenteric border, and the sheet preparations were fixed between the Ussing-type half chambers. Non-polarizable electrodes consisting of Zn/ZnSO$_4$ cells were used for passing DC current across the preparations. Polyethylene tube bridges filled with 1 M Tris-H$_2$SO$_4$/2% agar were used to connect the cells and the chambers. The PD$_t$ was recorded as described above. The fluid resistance between these PD-recording electrodes was determined in the absence of the tissue, and taken into consideration in the determination of $I_{sc}$. The bathing solutions of various Na$^+$ concentrations were prepared by replacing Na$_2$SO$_4$ with D-mannitol without changing the osmolality of the solutions. In the measurements of $I_{sc}$, the ionic composition of both mucosal and serosal bathing solutions was always identical. Amino acids were added to the mucosal solution. Preliminary experiments showed that any change in PD$_t$ was not evoked by the serosal administrations of acidic amino acids. Each measurement of $I_{sc}$ was carried out using a freshly prepared specimen.

RESULTS

The characteristics of intestinal transport of acidic amino acids

As the cellular uptakes of L-glutamate and L-aspartate by guinea pig ileum linearly increased with time up to 2 min (data not shown), 1 min incubation was adopted to determine the initial influxes across the brush border membrane.
The influx measurements by such a short period of incubation have an advantage of minimizing the influence of the cellular metabolism because acidic amino acids are rapidly transaminated (Neame and Wiseman, 1957; Parsons and Volman-Mitchell, 1974).

The unidirectional influxes of both L-glutamate and L-aspartate conformed to single Michaelis-Menten kinetics (Fig. 1), having values of maximum velocity of influx (J_{max}): \(31.9 \pm 3.9\) and \(31.7 \pm 2.1\) nmol·min^{-1}·cm^{-2} (n=6) for L-glutamate and L-aspartate, respectively. The values of half saturation concentration (K_i) were \(0.56 \pm 0.09\) mM for L-glutamate and \(0.35 \pm 0.04\) mM for L-aspartate. Figure 1 shows the inhibition kinetics between L-glutamate and L-aspartate which indicates the presence of the mutual inhibition of transport and nature of competitive type of inhibition. The values of inhibitor constant (K_i) obtained from this inhibition kinetics agree very well with the values of K_i described above. The values of K_i were \(0.46\) mM for L-glutamate and \(0.33\) mM for L-aspartate. The results of transport kinetics, together with the inhibition experiments, strongly suggested the common carrier mechanism for both amino acids.

Effect of acidic amino acid on the PD_i

Figure 2 (left panel) was a typical record of change in PD_i (∆PD) induced by the addition of L-glutamate to the mucosal bathing solution. As shown in this figure, the amino acid evoked a sudden increase in mucosal negativity just like the cases of addition of D-aldohexose or neutral amino acids (Igarashi et al., 1976; Himukai et al., 1983). The ∆PD was concentration-dependent and saturable.
The double reciprocal plot of 4PD and amino acid concentration revealed that APD obeyed single Michaelis-Menten kinetics (Fig. 2, right panel). Moreover, the value of maximum change in PD \( t \) was nearly equal (3.8 mV) for L-glutamate, L-aspartate, and D-aspartate. The mean values of \( K_t \) obtained from the electrical measurements (\( n=5 \)) were 0.41 mM for L-glutamate, 0.29 mM for L-aspartate, and 0.83 mM for D-aspartate. Furthermore, electrically estimated values of \( K_t \) for L-glutamate and L-aspartate were about the same as those estimated from influx measurements. This indicates that the 4PD evoked by the amino acids were linearly related to their influxes.

Increase in short-circuit current (\( \Delta I_{se} \)) induced by L-glutamate

In the next series of experiments, the relationship between amino acid-induced current flow and the concentration of Na\(^+\) was studied. For this purpose, \( I_{se} \) across the intestinal wall was measured at various Na\(^+\) concentrations ([Na\(^+\)]) because PD \( t \) was greatly influenced by the change in electrical conductivity of the incubation medium (HosHI et al., 1976b). The concentration of L-glutamate in the mucosal medium was fixed at 1 mM throughout this series of experiments.

Figure 3A shows the relationship between L-glutamate-induced \( \Delta I_{se} \) and [Na\(^+\)]. This figure indicates that the \( \Delta I_{se} \) is absolutely dependent on the presence
M. HIMUKAI

of medium Na+, and that the relationship between ΔI_{sc} and [Na\(^+\)] is not Michaelis-Menten type but sigmoid. For comparison, the relationship between the influx of L-glutamate and [Na\(^+\)] is also shown in Fig. 3B. The features are essentially the same as those shown in Fig. 3A. This seems to be strong evidence for the cotransport of L-glutamate and Na\(^+\).

The sigmoid relationship thus observed suggests that more than 1 Na\(^+\) are transported with a molecule of L-glutamate, and such excess Na\(^+\) transport may explain the electrogenicity of the transport. The relationship described above was analyzed by two different methods, GARAY and GARRAHAN's method (1973) and Hill analysis. In the former, it is assumed that multiple binding sites on a single transporter have the same affinity constant, and that there is no mutual interaction between the sites. It is also assumed that the translocation event occurs only when all sites are occupied. The kinetic equation derived from the basis of Garay and Garrahan's model is as follows:

\[
\frac{[\text{Na}\(^+\)]}{I^{1/n}} = \frac{K_{Na}}{I_{max}} + \frac{[\text{Na}\(^+\)]}{I_{max}^{1/n}}
\]

where I is the L-glutamate-induced ΔI_{sc}, K_{Na} the dissociation constant of the binding reaction of Na\(^+\), I_{max} the maximum value of ΔI_{sc} against [Na\(^+\)], and n the number of Na\(^+\) sites. According to Eq. (1), a plot of [Na\(^+\)]/I^{1/n} against [Na\(^+\)] will yield a straight line with a slope of 1/I_{max}^{1/n}, and the intercept at the abscissa corresponds to −K_{Na} if the number of Na\(^+\) sites per transporter is n. Figure 4A shows the plot of the data shown in Fig. 3A. As shown in this figure, the plot

Fig. 3. (A) L-Glutamate dependent increases in short-circuit current and (B) L-glutamate influxes as a function of the concentration of Na\(^+\) in the incubation medium. Reduction of Na\(^+\) concentration was carried out by replacing with D-mannitol without changing the osmolality. The concentration of L-glutamate was fixed at 1 mM.
of $[\text{Na}^+] / I^{1/n}$ against $[\text{Na}^+]$ became straight when $n$ was taken to be 2. From the linear relationship, the value of $K_{\text{Na}}$ and $I_{\text{max}}$ could be estimated to be 7.1 mM and 13.6 nmol·min$^{-1}$·cm$^{-2}$, respectively. Figure 4B shows a similar analysis for the data of L-glutamate influxes. In the case of Na$^+$/L-glutamate co-transport, the equation derived from Garay and Garrahan’s model is essentially the same as Eq. (1). Therefore, in order to describe the relationship between the influx of L-glutamate and $[\text{Na}^+]$, $I$ in Eq. (1) was substituted for by $V$ (the influx of L-glutamate). Similarly, $I_{\text{max}}$ in Eq. (1) was substituted for by $V_{\text{max}}$ (the maximum influx of L-glutamate against $[\text{Na}^+]$). Also in this case, a best fit for the data was obtained when $n=2$. The values of $K_{\text{Na}}$ and $V_{\text{max}}$ were estimated to be 5.2 mM and 15.3 nmol·min$^{-1}$·cm$^{-2}$, respectively.

In the latter method, i.e. the Hill plot analysis, it is assumed that cooperative binding between multiple Na$^+$ binding sites takes place. In this analysis, independent estimates of $I_{\text{max}}$ and $V_{\text{max}}$ were necessary. The author employed the values estimated from the Garay and Garrahan analysis as described above. The data shown in Fig. 3 were plotted in a log-log scale according to the logarithmic form of the Hill equation (Segel, 1975). As shown in Fig. 5, the points are scattering around the curve or line. The dotted lines were drawn assuming that the log-log relationship would be straight linear at $V=(1/2)V_{\text{max}}$. In this case, the Hill coefficients were estimated to be 1.2 (Fig. 5A) and 1.4 (Fig. 5B). The relationship appeared to be curvilinear, which may suggest the presence of the special case of allosteric interaction as described by Haber and Løeb (1983). However, the results obtained in the present study were not decisive.
The possibility of participation of the other cations in the Na⁺/L-glutamate co-transport across the mucosal border of guinea pig intestine

In the everted intestine, L-glutamate transport was not stimulated by H⁺ because it showed the optimum pH at about 7 when examined in the range of pH from 5.4 to 8.4 (Fig. 6) and was unaffected by the total replacement of K⁺, Ca²⁺, or Mg²⁺ by Na⁺ in the mucosal solution. Therefore, it is likely that Na⁺ is the sole cation involved in the transport of acidic amino acid in guinea pig small intestine.

Serosal appearance of L-glutamate across guinea pig ileum

As L-glutamate would be present in the intracellular fluid with a negative charge, outward directed electrochemical driving force may facilitate the exit tran-
Nat/ACIDIC AMINO ACID CO-TRANSPORT

port across the basolateral membrane. However, it has been shown that acidic amino acids, although a marked species difference was observed (Parsons and Volman-Mitchell, 1974), were rapidly transaminated intracellularly and changed to L-alanine (Neame and Wiseman, 1957; Burstoun et al., 1972). These observations indicate that the majority of acidic amino acid is then transported across the basolateral membrane by the transport carrier for neutral amino acid.

Table 1 shows the results of the increase in serosal appearance of amino acids after the incubation with L-glutamate. Major amino acids increased were L-glutamate and L-alanine. The increase of L-glutamate appearing in the serosal fluid was much greater than that of L-alanine in guinea pig intestine. In order to examine whether the negatively charged amino acid is transported across the basolateral membrane via the exchange mechanism with HCO₃⁻ or not, the effect of HCO₃⁻, on the transfer of L-glutamate was examined. As shown in Table 1, the presence or absence of HCO₃⁻ in the incubation medium did not alter the transfer of L-glutamate in the standard buffer solution (HCO₃⁻ free). Similarly, SITS (4-acetamido-4'-isothiocyanostilbene-2,2'-disulfonic acid; an inhibitor of HCO₃⁻-Cl exchange transport) was also without effect on the L-glutamate transfer.

DISCUSSION

The results of the present study clearly indicate the presence of a rheogenic Na⁺/acidic amino acid co-transport system in the everted intestine of guinea pig. The transport of both L-glutamate and L-aspartate was absolutely dependent on the presence of Na⁺ in the mucosal solution. Also, the initial influxes of these amino acids obeyed single Michaelis-Menten kinetics, indicating the presence of

Vol. 34, No. 5, 1984
a single binding site for the organic substrate on the carrier. The carrier affinities of L-glutamate and L-aspartate were higher than those observed in intestinal or renal transport of neutral amino acids (Lerner and Steinke, 1977; Himukai et al., 1978; Himukai and Hoshi, 1980; Samarzija and Frömter, 1982a).

The question of exact stoichiometry of transport of Na⁺ and acidic amino acid remains unsolved. There is a discrepancy with the results from the recent studies in brush border membrane vesicles. Several investigators have shown that the uptake of acidic amino acids into brush border membrane vesicles of intestine (Corcelli et al., 1982; Corcelli and Storelli, 1983) or kidney (Schneider et al., 1980; Schneider and Sacktor, 1980; Nelson et al., 1983) is markedly stimulated by Na⁺. They also found that this Na⁺/acidic amino acid co-transport was electroneutral because the uptake did not respond to the changes in membrane potentials. Moreover, Schneider and Sacktor (1980) showed, using membrane potential-sensitive fluorescent dye: 3,3'-dipropylthiodicarbocyanine iodide, that the co-transport of Na⁺ and glutamate by renal brush border membrane vesicles of rabbit did not cause an increase in fluorescence, i.e. did not depolarize the membrane. On the other hand, Burckhardt et al. (1980) reported the opposite conclusion, that the Na⁺/L-glutamate co-transport by the brush border membrane vesicles from rat renal proximal tubules was sensitive to membrane potential in the presence of intravesicular K⁺. They also demonstrated by an experiment using cyanine dye that net positive charge movement was involved in the co-transport. More recently, Nelson et al. (1983) showed the H⁺-Na⁺-glutamate co-transport system in the renal brush border membrane vesicles of rabbit and proposed a kinetic model that Na⁺, H⁺, and glutamate were co-transported and K⁺ (or H⁺ in the absence of intravesicular K⁺) was counter-transported in a 1:1:1:1 stoichiometry, so that the transport was electroneutral. The reason for the variance reported has yet to be described.

Although many studies using brush border membrane vesicles of mammalian kidney or intestine showed that the transport of acidic amino acid across the brush border membrane was electroneutral as described above, the transport in the intact epithelial cells of renal proximal tubule (Samarzija and Frömter, 1982b) and intestine (Schultz and Zalusky, 1965) of mammals was rheogenic. The characteristics of intestinal transport of acidic amino acid in guinea pig were very similar to the results examined electrophysiologically at the luminal membrane of rat renal proximal tubule by Samarzija and Frömter (1982b). In the present study, kinetic analysis for the sigmoid relationship between L-glutamate influxes or L-glutamate-induced ∆Iₑ and Na⁺ concentration indicated that 2 Na⁺ were co-transported with the amino acid across the brush border membrane.

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Na+/ACIDIC AMINO ACID CO-TRANSPORT

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