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THE ROLE OF POTASSIUM AND CHLORIDE IONS ON THE Na^+/ACIDIC AMINO ACID COTRANSPORT SYSTEM IN RAT INTESTINAL BRUSH-BORDER MEMBRANE VESICLES

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The Na^+/L-glutamate (L-aspartate) cotransport system present at the level of rat intestinal brush-border membrane vesicles is specifically activated by the ions K^+ and Cl^-. The presence of 100 mM K^+ inside the vesicles drastically enhances the uptake rate and the transient intravesicular accumulation (overshoot) of the two acidic amino acids. It has been demonstrated that the activation of the transport system depended only in the intravesicular K^+ concentration and that in the absence of any sodium gradient, an outward K^+ gradient was unable to influence the Na^+/acidic amino acid transport system. It was also found that Cl^- could specifically activate the Na^+-dependent L-glutamate (L-aspartate) uptake either in the presence or in the absence of K^+. Also the effect of Cl^- was observed only in the presence of an inward Na^+ gradient and it was noted to be higher when chloride ion was present on both sides of the membrane vesicles. No influence (activation or accumulation) was observed in the absence of the Na^+ gradient and in the presence of chloride gradient. L-Glutamate uptake measured in the presence of an imposed diffusion potential and in the presence of K^+ or Cl^- did not show any translocation of net charge.

Introduction

The fundamental role played by the sodium electrochemical gradient for the uptake of sugars, amino acids, neurotransmitters, organic and inorganic ions into the cells has been widely demonstrated with studies using plasma membranes isolated from different tissues [1–4].

On the other hand, by using membrane vesicles obtained from rat brain synaptosomes, the important role of potassium and chloride ions has been recognized in the uptake of the amino acids L-glutamate, glycine and other neurotransmitters. It has been demonstrated that these neurotransmitters are translocated by Na^+-dependent mechanisms and that these transport systems are activated or fully depend on the presence of potassium ions inside the vesicles or (and) the presence of chloride ions outside the vesicles [3–7].

Recently, the importance of potassium and chloride ions for the translocation of L-glutamate and glycine at the level of kidney proximal tubule and intestinal mucosal absorbing cells has been described. Burckhardt et al. [8] and Schneider and Sacktor [9] have reported that in brush-border membrane vesicles isolated, respectively, from rat and rabbit, the Na^+/glutamate cotransport is stimulated and energized by the presence of K^+ inside the vesicles. Bogè and Rigal [10] using brush-border membrane vesicles isolated from the

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Abbreviations: Tris, tris(hydroxymethyl)aminomethane; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

mediterranean teleost *Boops salpa,* have characterized a Na^+-dependent glycine transport, which requires chloride ions in the extravesicular space.

Using isolated membrane vesicles, we have recently demonstrated that a Na^+/L-glutamate (or L-aspartate) cotransport system is present at the level of rat intestinal brush border [2].

The results presented in this report indicate that also in the intestinal brush-border membrane preparation both potassium and chloride ions play an important role in stimulating the Na^+-dependent transport system of the two acidic amino acids.

Materials and Methods

Chemicals. L-[2,3-^{3}H]Glutamic acid, L-[2,3-^{3}H]aspartic acid were obtained from New England Nuclear, Boston, MA, U.S.A.; all chemicals used during the study were of analytical grade purity.

Preparation of membrane vesicles. Brush-border membrane vesicles were isolated by the $CaCl_2$ precipitation method, as previously described [2]. The membrane vesicles used for the uptake, unless stated otherwise, were suspended in a buffered solution containing 100 mM mannitol, 20 mM Hepes-Tris (pH 7.5).

The composition of the internal and external medium of the membrane vesicles was modified to investigate the effect of different ions on the uptake of the acidic amino acids. A preincubation of the vesicles with salts for 1 h at 0°C was considered sufficient to preequilibrate the different salts through the membrane vesicles.

The protein concentration was about 10 mg/ml in the final suspension.

Uptake studies. Uptakes of L-[^{3}H]glutamate, L-[^{3}H]aspartate were carried out as previously described [2]. Unless stated otherwise, 10 μl of membrane suspension were added to 90 μl of incubation medium kept at 25°C. The composition of the incubation media will be indicated in the legends of the figures.

The uptake was stopped by directly pipetting, at selected time intervals, 20 μl of the incubation mixture on a membrane filter (0.45 μm pore size, Millipore, Bedford, MA, U.S.A.).

The filters were immediately rinsed with 5 ml of ice-cold stop solution containing 100 mM mannitol, 5 mM Hepes-Tris (pH 7.5), 100 mM NaCl, 100 mM choline chloride and 0.5 mM $HgCl_2$. In some experiments the composition of the stop solution was modified to balance the osmolarity on the incubation media.

Membrane free incubation media were handled as blanks in an identical manner. Radioactivity retained by the filters was analyzed by standard liquid-scintillation procedures. The radioactivity of the filters containing membranes was at least 3-times higher than that of the blanks.

All experiments presented in this paper were repeated at least three times, and were always performed in triplicate; as previously discussed [2] only results of typical experiments are shown.

Data are expressed as pmol/mg of protein and are means ± S.D. of the triplicates.

In the experiments performed to illustrate the effect of K^+ on the Na^+ dependent acidic amino acid uptake, an identical Cl^- concentration in the intra- and extravesicular spaces was used.

Enzyme assay. Membrane purification was routinely checked as previously described [2].

Protein determination. Membrane protein was determined by the method of Bradford [11], using the Bio-Rad kit (Richmond, CA, USA) and bovine gamma globulin as a standard.

Results

The effect of intravesicular K^+ on the uptake of L-glutamate and L-aspartate

Fig. 1(a and b) shows that in the presence of an inwardly directed Na^+ gradient, the L-glutamate and L-aspartate uptake are strongly stimulated by the presence of an outwardly directed potassium gradient (100 mM in/10 mM out).

The stimulating effect of internal K^+ reaches its maximum in the first minutes of incubation, while after that time the effect is much smaller and after 60 min the same equilibrium values are reached in all the experimental conditions.

Fig. 1(a and b) also shows that a similar stimulating effect was obtained when, in the presence of the same Na^+ gradient, 100 mM potassium was present on both sides of the membrane vesicles.

Fig. 2 shows that in the presence of a Na^+ gradient (out > in) the stimulating effect of potassium depended on the internal concentration of

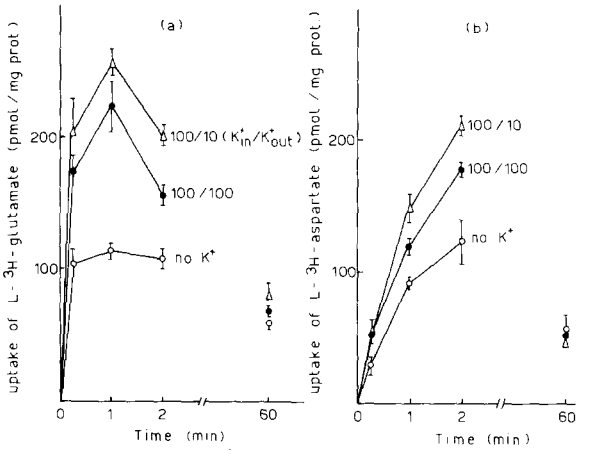

Fig. 1. Effect of K^+ on the Na^+ dependent uptake of L-glutamate (a) and L-aspartate (b). Membranes were preloaded with 100 mM KCl plus 100 mM choline chloride (membrane type 1) or with 200 mM choline chloride (membrane type 2). At zero time, in all the experimental conditions the external medium contained 100 mM mannitol, 20 mM Hepes-Tris (pH 7.5), 0.1 mM L-[^{3}H]glutamate (or L-[^{3}H]aspartate), 100 mM NaCl; in addition 100 mM choline chloride plus 10 mM KCl (△) or 100 mM KCl (●) were present with membrane type 1; 100 mM choline chloride (○) with membrane type 2.

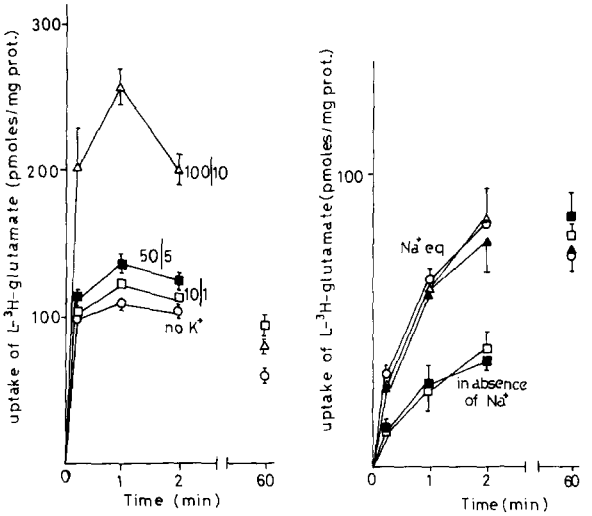

Fig. 2. Dependence of the K^+ effect on the L-glutamate uptake by the internal cation concentration. Membranes were preloaded with 100 mM KCl plus 100 mM choline chloride (△), 50 mM KCl plus 150 mM choline chloride (■), 10 mM KCl plus 190 mM choline chloride (□), 200 mM choline chloride (○). In all cases at zero time the composition of the external medium of the vesicles was 100 mM mannitol, 20 mM Hepes-Tris (pH 7.5), 0.1 mM L-[^{3}H]glutamate, 100 mM NaCl, 10 mM choline chloride plus different amounts of KCl as indicated in the figure.

the cation. In the experiments shown in this figure, the same potassium concentration ratio was maintained but the potassium concentration inside the vesicles was lowered. It can be seen that, when internal potassium concentration was 10 mM, the stimulating effect was almost abolished. The results shown in Fig. 1(a and b) and Fig. 2 are consistent with the notion that the stimulating effect of internal K^+ is related not to its transmembrane (internal/external) gradient but rather to its internal concentration per se.

To shed light on whether in the absence of the Na^+ gradient (or in complete absence of Na^+) the outwardly directed K^+ gradient could energize the transport and the accumulation of L-glutamate and L-aspartate inside the vesicular space, experiments were carried out in the absence of sodium gradient ($[Na^+]_{in} = [Na^+]_{out}$) or in a sodium-free medium.

Fig. 3 shows that in both experimental conditions the potassium gradient (in > out) is unable to affect the uptake rate of the Na^+-dependent L-glutamate (L-aspartate) transport system.

Fig. 4 shows that the degree of the stimulating

Fig. 3. The effect of the internal K^+ on the L-glutamate uptake in the absence of Na^+ or in the presence of an equal Na^+ concentration in the extra- and intravesicular media. In the group of experiments in which equal concentration of Na^+ on both sides of membranes were present (○, △, ▲), the membranes were preloaded with 100 mM NaCl plus 100 mM KCl (△, ▲) or 100 mM NaCl plus 100 mM choline chloride (○). At the start of uptake the following intravesicular and extravesicular ionic media concentration (expressed as mM) were obtained; in all cases $[Cl^-]_{in} = [Cl^-]_{out} = 200$; $[Na^+]_{in} = [Na^+]_{out} = 100$, in addition $[K]_{in}/[K]_{out} = 100/100$ (△) or $[K]_{in}/[K]_{out} = 100/10$ (▲) or $[choline]_{in}/[choline]_{out} = 100/100$ (○). A second group of experiments was performed using membranes in the absence of Na^+ so that the following intra and extravesicular salts concentration were present at the start of incubation. In both cases $[Cl^-]_{in} = [Cl^-]_{out} = 200$, $[choline]_{in} = [choline]_{out} = 100$, in addition $[K^+]_{in}/[K^+]_{out} = 100/100$ (□) or $[K^+]_{in}/[K^+]_{out} = 100/10 + 90$ mM external choline (■). In the different experimental conditions, the extravesicular medium contained 0.1 mM L-[^{3}H]glutamate, 100 mM mannitol, 20 mM Hepes-Tris (pH 7.5).

effect of potassium is related to the extent of the Na^+ gradient. At 50 mM Na^+-gradient (out > in), the stimulating effect of internal 100 mM potas-

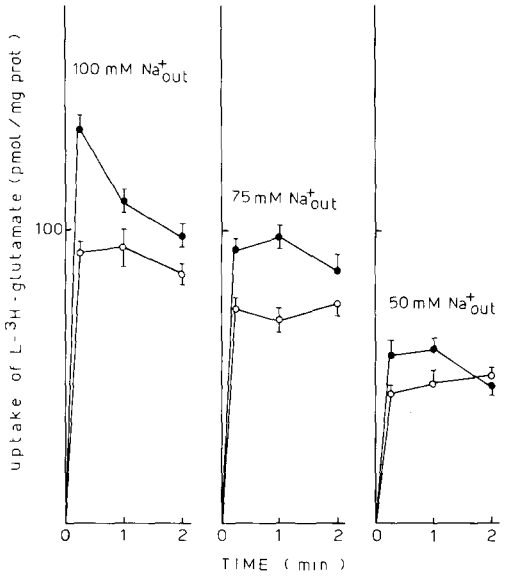

Fig. 4. Dependence of the K^+ effect on the extent of the Na^+ gradient. Membranes were preloaded with 100 mM KCl plus 100 mM choline chloride. At zero time the following external medium composition were obtained: 100 mM mannitol, 20 mM Hepes-Tris (pH 7.5), 0.1 mM L-[^{3}H]glutamate, 10 mM KCl; in addition 100 mM NaCl (a), 75 mM NaCl (b), 50 mM NaCl (c) plus different amounts of choline chloride to balance the osmolarity.

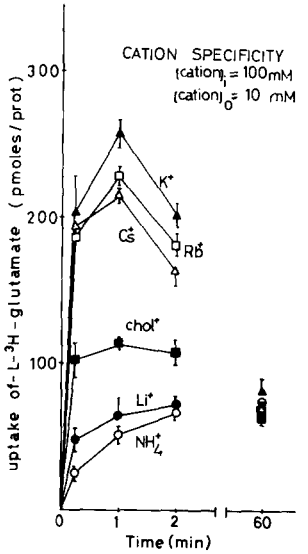

Fig. 5. Specificity of the internal cation for the stimulation of L-glutamate uptake. Membranes were preloaded with 200 mM choline chloride or with 100 mM choline chloride plus 100 mM of the other monovalent cations indicated in the figure so that when the uptake was started, an outwardly directed gradient of the different cations was present. The composition of the final incubation medium was 100 mM mannitol, 20 mM Hepes-Tris (pH 7.5), 0.1 mM L-[^{3}H]glutamate, 100 mM NaCl, 100 mM choline chloride and 10 mM of chloride salts of the different cations.

sium is low, but still measurable for L-glutamate; it is absent for L-aspartate (data not shown). The experiments shown in Fig. 3 and Fig. 4 strongly suggest that the acidic amino acid transport system has an absolute requirement for the Na^+ electrochemical gradient (out > in) and that the K^+ gradient cannot be used as a driving force to accumulate the amino acids inside the vesicles.

Concerning the specificity of K^+ in stimulating the Na^+-dependent L-glutamate (L-aspartate) uptake, the results reported in Fig. 5 indicate that only Cs^+ and Rb^+ can partially substitute K^+, while in the presence of choline ions, Li^+ or NH_4^+ the measured uptake values were much lower.

The effect of the chloride ion on the L-glutamate and L-aspartate uptake

The role of the chloride ion was studied measuring the uptake of L-glutamate and L-aspartate in the presence of an inward sodium gradient, using sodium salts with different anions.

It can be noted (Fig. 6a and 6b) that the

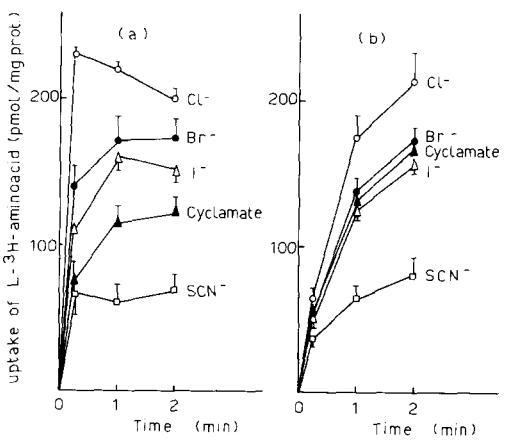

Fig. 6. Anion specificity on the stimulation of the Na^+ dependent L-glutamate and L-aspartate uptakes. Membranes were isolated in 300 mM mannitol, 20 mM Hepes-Tris (pH 7.5). The final external incubation medium contained 100 mM mannitol, 20 mM Hepes-Tris (pH 7.5), 0.1 mM L-[^{3}H]glutamate (or L-[^{3}H]aspartate), 100 mM NaCl or other sodium salts indicated in the figure. The equilibrium values were the same for all the time courses; the medium value was 120 pmol/mg protein.

substitution of the external chloride ion drastically reduces the uptake rates of the two amino acids. The most effective inhibitory anion was SCN^- while in the presence of the bromide or iodide ion the transport system partially restored its activity and its concentrative ability.

The results shown in Fig. 7a give further information on the importance of the external chloride concentration. It can be seen that in the presence of 200 mM chloride on both sides of the membrane vesicles the sodium dependent L-glutamate uptake is much more efficient if compared with the uptake measured when 100 mM external chloride were substituted by nitrate or gluconate ($[Cl^-]_{in} > [Cl^-]_{out}$).

When the same experiments shown in Fig. 7a were carried out in the presence of an outward potassium gradient, a significant increase of L-glutamate uptake was observed in all the experimental conditions, but again, a drastic decrease in the uptake values was found when the external chloride concentration was reduced to half. The results of Fig. 7b indicate that both (internal) potassium and chloride ions are required to fully

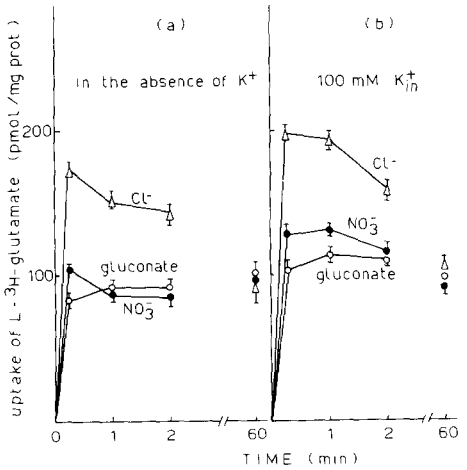

Fig. 7. The effect of the substitution of the external Cl^- on the Na^+ dependent L-glutamate uptake in the absence (a) or in the presence of internal K^+ (b). Membranes were preloaded with 200 mM choline chloride (a) or 100 mM choline chloride, 100 mM KCl (b). In both cases, the composition of external medium was 0.1 mM L-[^{3}H]glutamate, 20 mM Hepes-Tris (pH 7.5), 100 mM mannitol, 100 mM choline chloride. In addition 100 mM NaCl (△) or $NaNO_3$ (●) or sodium gluconate (○) were present and, when membranes b were used, 10 mM KCl.

activate the sodium dependent L-glutamate transport system. Furthermore, the results shown in Fig. 7(a and b) do not give any quantitative information on the chloride concentration required by the transport system, but give evidence of the importance of a high external chloride concentration in activating the sodium-dependent L-glutamate uptake. Finally, the data shown in Figs. 6 and 7 favour the view that the chloride ion is important on the outer side of the membrane but also do not exclude a role for this ion inside the membrane.

To answer the question on which side of the membrane the chloride ion interacted with the transport system and whether a chloride gradient could energize the accumulation of the amino acids in the intravesicular space, experiments were carried out measuring the uptake rates in the presence of an inward chloride gradient and of an equal concentration of sodium on both sides of the membranes (Fig. 8a). In the same figure (8a) are reported the L-glutamate uptakes measured in the presence of the same concentration of chloride on both sides of the membrane, in the presence of an outward chloride gradient and in the complete absence of chloride. Each time chloride was substituted, it was substituted for gluconate. It can be noted that in the absence of a sodium electrochemical gradient the L-glutamate uptake was completely unaffected by the presence of chloride or chloride gradient.

Interestingly, the substitution of chloride or gluconate for thiocyanate on one or both side of the membrane vesicles significantly decreased the L-glutamate uptake (data not shown).

On the other hand, when L-glutamate uptake was measured in the simultaneous presence of an inward sodium gradient and an outward potassium gradient (Fig. 8b), the specificity of the activating role of chloride could be clearly established.

As shown in Fig. 8b, when the effect of the presence of equal concentration of chloride or gluconate on L-glutamate uptake were compared, a faster uptake rate and a larger accumulation ability (overshoot) were observed when the chloride ion was present on both sides of the membranes. In the same figure, it can also be seen that when chloride is present only on one side (outside or

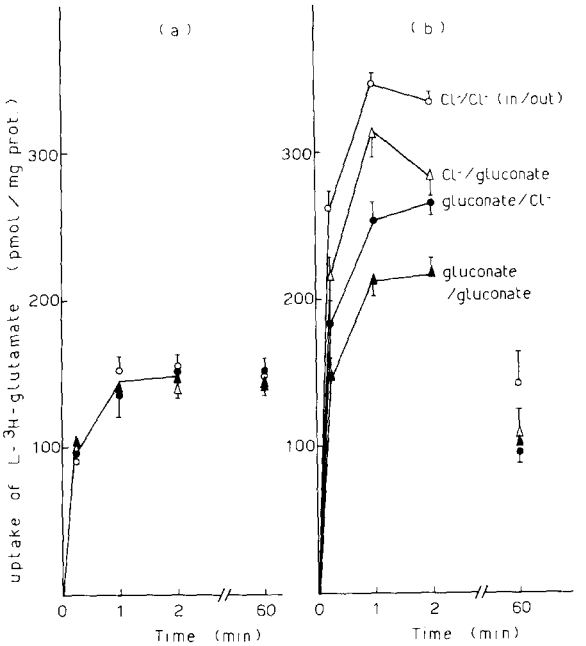

Fig. 8. The effect of an outward or inward chloride gradient on the Na^+ dependent L-glutamate uptake. Membranes were preloaded with 100 mM KCl, potassium gluconate, NaCl or sodium gluconate. The composition of the external medium was 100 mM mannitol, 20 mM Hepes-Tris (pH 7.5), 0.1 mM L-[^{3}H]glutamate plus 100 mM NaCl (○, ●) or sodium gluconate (△, ▲). The effect of an outward (△) or inward (●) chloride gradient or of the presence of an equal chloride concentration on both sides of the membrane (○) was studied in the presence of equal concentration of Na^+ on both sides of membranes (a) or in the presence of an 100 mM Na^+ inward gradient (b). The membranes of the experiments shown in (a) were preloaded with 100 mM NaCl (○, △) or sodium gluconate (●, △); those of the experiments shown in (b) were preloaded with 100 mM KCl (○, △) or potassium gluconate (●, ▲).

inside) of the membrane, the uptake mechanism in both cases is faster and more efficient (larger overshoot) if compared with that in which chloride is substituted for gluconate on both sides; but slower and less efficient than when chloride is present on both sides of the membrane vesicles.

These data, together with those reported in Fig. 8a, strongly suggest that (a) the chloride ion has a specific stimulating effect on the sodium dependent L-glutamate uptake present at the level of the intestinal brush-border membrane vesicle, (b) this effect can be observed only in the presence of a Na^+ gradient, (c) the effect can be observed in the presence or in the absence of internal K^+, (d) the system can be stimulated by the presence of chloride on the internal or on the external side of the membrane, (e) to fully activate the transport system, the chloride ion must be present on both sides of the membrane.

Discussion and Conclusions

The data reported in this paper clearly demonstrate the important role of potassium and chloride ions in the translocation of the acidic amino acids L-glutamate and L-aspartate through the rat intestinal enterocyte brush-border membrane vesicles.

It has been established that the sodium dependent L-glutamate (L-aspartate) transport system located on these membranes can be specifically activated by the presence of the potassium ion inside (Figs. 1 and 4) or (and) by the presence of the chloride ion on one or both sides of the membrane vesicles (Fig. 8b).

The two ions can separately influence the transport system and their effect can be added (Fig. 7 a and b).

Finally, the activation of the transport system by the two ions is strictly dependent on the presence of an inward sodium gradient, i.e. in the presence of an equal concentration of sodium on both sides of the membrane even the simultaneous presence of an outward potassium gradient and an inward chloride gradient through the membranes were not able to affect (energize or stimulate) the transport system (Fig. 3 and 8a).

From the data in the literature available until now, it is evident that the ways in which the two ions can interact are extremely variable and the answers regard mainly the following questions: (i) the side interaction; (ii) the question if only one or both ions are required; (iii) the role which they play in the translocation mechanism.

As regards the requirement of potassium ion, the observation we have reported that it must be present on the internal side of the membrane vesicles is in agreement with those reported by other authros [4,7–9,12].

On the other hand, the ability of chloride in stimulating sodium transport system was reported to be generally on the external side [3,6,10,13].

Only the efflux of L-glutamate by the synaptic

plasma membrane vesicles was found to be stimulated either by internal and external chloride [7].

The influence (activation, energization) of sodium dependent transport system has mainly been found to be sustained by one of the two ions [3,4,6–10]. Requirements for both potassium and chloride ions have been observed by Nelson and Rudnick [13] on platelets serotonin transport. One of the most intriguing but at the same time interesting points to establish is certainly the role played by the two ions in the translocation mechanism. Until now two possibilities have been taken into account: (a) the possibility that the ions could interact with the transport system changing its conformation without being translocated; (b) the ions could be cotransported or countertransported and could provide the energy for accumulating the substrate inside the vesicles.

Burckhardt et al. [8] in rat and Schneider and Sacktor [9] in rabbit kidney cortex membrane vesicles found that an outward potassium gradient was able to accumulate L-glutamate inside the vesicles. Similar results were found by Nelson and Rudnick [12] studying serotonin uptake in platelets.

On the other hand the neuronal transport (reuptake) of some neurotransmitters such as γ-aminobutyric acid [3], glycine [6], the transport of glycine into erythrocyte [14], serotonin into platelets [13] require in all cases chloride and in all cases the neutrotransmitters can be accumulated by the sole chloride electrochemical gradient. These experimental evidences can support the hypothesis that the cotransport of sodium and the substrate could be directly linked to a cotransport of chloride or countertransport of potassium (or both at the same time), but do not represent a final demonstration of this mechanism.

More direct evidences in favour of such a mechanism come from efflux measurements as reported by Kanner and Marva [7] and Nelson and Rudnick [13].

On the basis of L-glutamic acid efflux measurements in synaptosomal membrane vesicles Kanner and Marva [7] conclude that the L-glutamate transporter catalizes sodium and L-glutamate cotransport while it simultaneously catalizes antiport of potassium. It should be noticed that this efflux was stimulated by the presence of either internal and (or) external chloride ion.

Nelson and Rudnick [13] draw the conclusion that the transport of serotonin into platelets membrane vesicles uses a sodium serotonin-chloride symport and a potassium (or hydrogen) ion antiport.

The only information available on the requirement of an additional ion (chloride) for a sodium dependent transport system in the intestine are on the glycine and 2-aminoisobutyric acid transport system described by Bogé and Rigal [10] in brush-border membrane vesicles isolated from the teleost *Boops salpa*.

At variance from the rat intestinal acidic amino acid transport system the fish intestinal sodium/amino acid cotransport system strictly depends on the presence of the external chloride ion. In agreement with our observation, in the presence of an inward chloride gradient but in the absence of a sodium gradient the transport system is unable to act as a driving force (to give rise to any overshoot phenomenon).

As previously pointed out, in rat intestinal brushborder membrane vesicles, in the absence of an inward sodium gradient, the presence of an outward potassium gradient or (and) an inward chloride gradient are unable to be used as driving forces for the accumulation of L-glutamate (L-aspartate). Moreover, no activation (increase of uptake rate) of the two ions on the transport system could be observed in the absence of a sodium gradient.

Another interesting point to be discussed is if the interaction of the sodium/acidic amino acid transport system with the potassium or chloride ion could change the cotransport mechanism into a rheogenic one. This possibility has been reported by Burckhardt et al. [8] for L-glutamate transport in kidney cortex brush-border membrane vesicles.

In a previous report we have concluded that the cotransport of L-glutamate and L-aspartate was carried out without translocation of net charge [2].

The following observations support the conclusion that the sodium/acidic amino acid transport system of the rat intestinal brush-border membrane also in the presence of the two ions remains unaffected by changes in membrane potential: (1) measurements of L-glutamate uptake in the presence of an inward sodium gradient, an outward

potassium gradient and in the presence of chloride on both sides of the vesicles in the absence or in the presence of valinomycin are identical [2]; (2) experiments of L-glutamate uptake (Fig. 7 a and b) in which the effect of the imposition of a gradient of anions of different permeability was measured, were unaffected by the presence of K^+.

Our observations favour the view that the role played by potassium and chloride ions does not imply translocation of any of these ions but rather an activation of the sodium/amino acid cotransport system at the level of conformational changes.

This 'double activated' uptake system could account for the much larger intravesicular accumulation (overshoot) observed for the two amino acids and could be related to a more efficient utilization of the Na^+ electrochemical gradient.

Experiment are in progress trying to investigate the influence of K^+ and Cl^- on the kinetic parameters of the Na^+/acidic amino acid cotransport system of the rat intestinal brush-border membrane vesicles.

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