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Uptake of Lysine and Proline *via* Separate α-Neutral Amino Acid Transport Pathways in *Mytilus* Gill Brush Border Membranes

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Summary. Brush border membrane vesicles (BBMV) were prepared from the gills of the marine mussel, *Mytilus edulis*. These membranes contained two distinct pathways for cotransport of Na^+ and α-neutral amino acids. The major pathway in mussel gill BBMV was the alanine-lysine (AK) pathway, which had a high affinity for alanine and for the cationic amino acid, lysine. The AK pathway was inhibited by nonpolar α-neutral amino acids and cationic amino acids, but was not affected by β-neutral amino acids or imino acids. The kinetics of lysine transport were consistent with a single saturable process, with a J_{max} of 550 pmol/mg-min and a K_t of 5 μM. The AK pathway did not have a strict requirement for Na^+, and concentrative transport of lysine was seen in the presence of inwardly directed gradients of Li^+ and K^+, as well as Na^+. Harmaline inhibited the transport of lysine in solutions containing either Na^+ or K^+. The alanine-proline (AP) pathway transported both alanine and proline in mussel gill BBMV. The AP pathway was strongly inhibited by nonpolar α-neutral amino acids, proline, and α-(methylamino)isobutyric acid (Me-AIB). The kinetics of proline transport were described by a single saturable process, with a J_{max} of 180 pmol/mg-min and K_t of 4 μM. In contrast to the AK pathway, the AP pathway appeared to have a strict requirement for Na^+. Na^+-activation experiments with lysine and proline revealed sigmoid kinetics, indicating that multiple Na^+ ions are involved in the transport of these substrates. The transport of both lysine and proline was affected by membrane potential in a manner consistent with electrogenic transport.

Key Words alanine transport · lysine transport · proline transport · brush-border membrane vesicles · Na-cotransport · *Mytilus edulis*

Introduction

Marine mussels, like all other soft-bodied marine invertebrates, absorb amino acids from seawater into the cells of their integument (Stephens, 1988). This process functions against extreme concentration gradients: from submicromolar amino acid concentrations in seawater into a cellular pool which, in mussels, is around 100 mM (Wright, Secomb & Bradley, 1987). Net amino acid uptake in mussels has been observed from concentrations as low as 10 nM (Manahan, Wright & Stephens, 1983), which represents transport against a concentration gradient greater than one millionfold. This transport may play an important role in the nutrition of these animals, and in the maintenance of the high intracellular free amino acid concentrations characteristic of their tissues (Wright, 1988).

Uphill transport of amino acids and other organic solutes in vertebrate epithelia, such as the small intestine and kidney, is coupled to the downhill movement of sodium, a process generally described by the Na-gradient hypothesis (Crane, 1962). It is now clear that the typical animal cell has several distinct Na^+-amino acid transporters (Christensen, 1984; Stevens, Kaunitz & Wright, 1984). These vertebrate transporters, however, do not normally move substrates against the extreme concentration gradients that exist across the brush border membrane of the mussel integument. Nevertheless, the mechanism of amino acid transport in marine mussels may resemble that in vertebrates, as amino acid uptake in intact mussel gills is sensitive to the presence of Na^+ in seawater (Wright, 1987).

The principal site of amino acid uptake in marine mussels is the gill (Pequignat, 1973). In a previous study, we used brush border membrane vesicles prepared from *Mytilus edulis* gills to study alanine transport (Pajor & Wright, 1987). The results showed that the uptake of alanine is coupled to the movement of Na^+, consistent with the sodium gradient hypothesis. It was also evident, however, that Li^+ and K^+ gradients could drive uphill transport of alanine. A study with intact mussel gills suggested that uptake of alanine involves at least two distinct pathways (Wright, 1985), one of which transports lysine and the other proline, in addition

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to alanine. This paper describes the results of a study using mussel gill brush border membrane vesicles (BBMV) to characterize the pathways for the transport of alanine, and, in particular, the effects of cation gradients on the two pathways.

Materials and Methods

Animals

Specimens of the common blue mussel (*Mytilus edulis*) were purchased from Sea Life Supply (Sand City, CA) and from the Bodega Marine Laboratory (Bodega Bay, CA). They were maintained in refrigerated aquaria (12°C) containing recirculating, aerated artificial seawater (Instant Ocean). Animals were not fed and were used within two weeks of collection.

Membrane Preparation

Gill brush border membrane vesicles (BBMV) were prepared by differential and sucrose density gradient centrifugation as described in a previous study (Pajor & Wright, 1987). Briefly, the gills were soaked in an artificial seawater containing a high concentration of K^+ (115 mM) and 1 mM dithiothreitol (DTT) in order to remove mucus. The gills were then homogenized in a buffer composed of 500 mM sorbitol, 5 mM ethyleneglycolbis(β-aminoethylether)-N-N'-tetraacetic acid (EGTA), 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid buffered to pH 7.6 with tris(hydroxymethyl)-aminomethane (HEPES-Tris). A crude brush border membrane fraction was isolated by several differential centrifugation steps. This fraction was purified using a linear 30 to 50% (wt/vol) sucrose density gradient. The purity of the final membrane preparation was assessed through the use of enzymatic membrane markers as well as amino acid transport. As shown previously (Pajor & Wright, 1987), the final brush border vesicle fraction had the highest enrichment (relative to the initial homogenate) of the apical membrane marker, γ-glutamyl transpeptidase (GGTP; 18-fold), the highest activity of cation-dependent alanine transport, and the lowest enrichment of the basal-lateral membrane marker, K-dependent paranitrophenyl phosphatase (PNPPase; 1.8-fold).

For most of the studies outlined here, the final brush border membrane fraction was resuspended as described previously (Pajor & Wright, 1987), in a buffer containing 600 mM mannitol and 10 mM HEPES-Tris at pH 7.6. In the experiments with vesicles containing KCl, the final membrane fraction was resuspended in 300 mM KCl, 10 mM HEPES-Tris at pH 7.6 and left on ice approximately 6 hr, followed by 30 min at room temperature.

Transport Measurements

The transport of substrates into membrane vesicles was measured using a rapid filtration method described by Wright et al. (1983). The transport reaction was run at room temperature, and stopped with 1 ml ice-cold isosmotic mannitol buffer (600 mM mannitol, 10 mM HEPES-Tris, pH 7.6). The reaction mixture was then rapidly filtered with suction through a 0.45 μm Millipore filter (HAWP), and washed with 4 ml of cold mannitol buffer. The labeled substrate retained on the filter was extracted in scintillation vials containing 6 ml scintillation cocktail (Betaphase), and radioactivity was determined using a liquid scintillation counter (Beckman LSC-3801). Counts were corrected for variable quench, and all uptakes were corrected for nonspecific binding to membranes and filters.

Chemicals

^{3}H-alanine (44-85 Ci/mmol), ^{3}H-lysine (80-98 Ci/mmol), and ^{3}H-proline (127 Ci/mmol) were purchased from NEN. All other chemicals were obtained from Sigma Chemical Corporation.

Results

Inhibition of Alanine Uptake by Lysine and Proline

Studies with intact gill tissue have shown that there are at least two pathways for the transport of alanine in the gills of marine mussels (Wright, 1985). Both of the pathways transport α-neutral amino acids, such as alanine, but they can be distinguished since one pathway shows a preference for cationic amino acids such as lysine, whereas the other shows a preference for proline. The presence of these two pathways in gill brush border membrane vesicles (BBMV) was confirmed by the results shown in Fig. 1. Unlabeled alanine (1 mM) inhibited approximately 98% of the total uptake of 0.5 μM ^{3}H-alanine. Proline, at 0.5 mM concentration, inhibited approximately 28% of the total alanine uptake, and increasing the proline concentration to 1 mM produced no further inhibition. Alanine uptake was reduced approximately 84% by 0.5 mM lysine. There was no further inhibition of alanine uptake when the concentration of lysine was increased to 1 mM. The combination of 0.5 mM proline and 0.5 mM lysine reduced alanine uptake more than millimolar concentrations of either proline or lysine alone.

Kinetics of Alanine Uptake

The results of the inhibition studies described above suggested that one way to study the two alanine transport pathways individually was to inhibit one of them with an excess of either proline or lysine. Figure 2 shows a representative experiment which illustrates the kinetics of alanine uptake through each of the pathways. As was seen earlier (Pajor & Wright, 1987), the kinetics of total alanine uptake could be described by the sum of a single saturable component and a diffusive pathway (Fig. 2). The apparent diffusive pathway might, however, represent a carrier-mediated pathway of low substrate affinity. In experiments with membranes from three

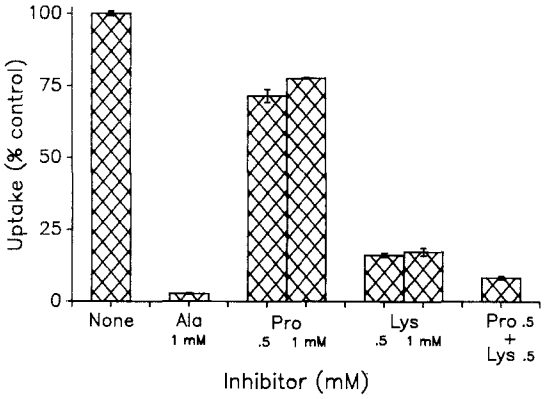

Fig. 1. Inhibition of alanine uptake by alanine, lysine, and proline. Vesicles contained 600 mM mannitol, 10 mM HEPES-Tris, pH 7.6. Transport buffers contained 0.5 μM ^{3}H-L-alanine, 260 mM NaCl, 10 mM HEPES-Tris, pH 7.6, and mannitol to maintain isosmolarity. Transport buffers also contained 0.5 or 1 mM alanine, lysine or proline as indicated in the figure. Ten-second uptakes were measured. The bars represent means ± SEM of triplicate determinations from a single membrane preparation

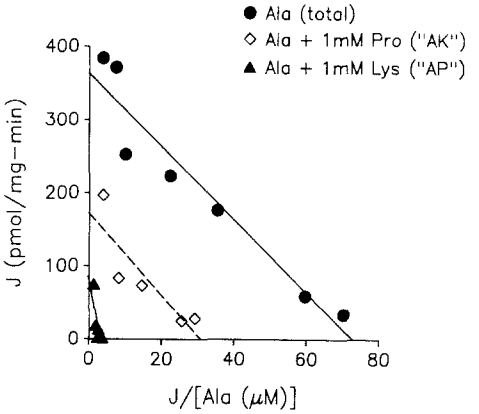

Fig. 2. Kinetics of alanine uptake. Woolf-Augustinsson-Hofstee plot of the initial rate (10 sec) of alanine uptake (J) at alanine concentrations ranging from 0.5 to 100 μM. Resuspension and transport buffers were the same as those in Fig. 1. The filled circles show the total uptake of alanine (corrected for a diffusive component of P = 14 nl/mg-sec), with a J_{max} of 365 pmol/mg-min and K_t of 5 μM. The open diamonds show the uptake of alanine via the AK pathway (determined using 1 mM proline to inhibit alanine uptake through the AP pathway) with J_{max} = 176 pmol/mg-min, and K_t = 6 μM. The filled triangles show the uptake of alanine via the AP pathway (determined using 1 mM lysine to inhibit the uptake of alanine through the AK pathway), with a J_{max} = 87 pmol/mg-min and K_t = 26 μM. The points represent means of triplicate determinations from a single membrane preparation

separate preparations, the mean J_{max} for total alanine uptake was 246 ± 60 pmol/mg-min (mean ± SEM), the apparent K_t was 4 ± 0.3 μM, and the apparent permeability coefficient, P, was 0.1 ± 0.03 nl/mg-sec.

The addition of 1 mM proline was used to block alanine uptake through the alanine-proline pathway (hereafter referred to as the "AP pathway") and allowed study of alanine uptake through the alanine-lysine (AK) pathway. In three experiments, the J_{max} for alanine transport through the AK pathway was 171 ± 4 pmol/mg-min (about 77 ± 15%, mean ± SEM, of the total alanine uptake), with an apparent K_t of 6 ± 0.1 μM. The uptake of alanine through the AP pathway was studied by using an excess of lysine to block alanine uptake via the AK pathway. In three separate experiments, the mean J_{max} for alanine uptake in the presence of 1 mM lysine was 77 ± 7 pmol/mg-min (33 ± 5% of the total alanine uptake), and the apparent K_t was 20 ± 3 μM. As it is likely that the AP and AK pathways were influenced to some extent by the presence of a 1 mM concentration of the competing substrate (e.g., the AP pathway may be inhibited by millimolar lysine), the kinetics of alanine transport through each should be considered estimates of the parameters, J_{max} and K_t, for each pathway.

Cation Selectivity of Alanine Transport

Our previous study of Na^+-alanine cotransport (Pajor & Wright, 1987) revealed an unexpected low specificity for Na^+. Uphill transport of alanine occurred in the presence of inwardly directed gradients of Li^+ and K^+, as well as Na^+. In this study, the effect of cations was examined separately for each of the alanine transport pathways. Figure 3 shows initial rates of alanine transport in the presence of different cations. In accord with our earlier results, total alanine uptake in the presence of Li^+ and K^+ was 40 and 16%, respectively, of that seen in Na^+ (Fig. 3), though it should be noted that the relative rates of uptake of alanine in the presence of these cations varied between membrane preparations. In Li^+ the mean uptake was 65 ± 8% (n = 7), and in K^+ the mean uptake was 35 ± 4% (n = 20) of the uptake seen in Na^+. The order of cation preference was always $Na^+ > Li^+ > K^+$.

The AK pathway appeared to account for the low cation selectivity seen with total alanine uptake. The initial rate of alanine uptake in the presence of 1 mM proline (to block the AP pathway) was 76% in Li^+ and 26% in K^+, relative to that measured in the presence of Na^+ (Fig. 3). In contrast, transport through the AP pathway was dependent upon the presence of sodium. The initial rate of alanine transport in the presence of 1 mM lysine (to block transport through the AK pathway) was reduced by more than 98% when Na^+ was replaced by Li^+ or K^+ (Fig. 3).

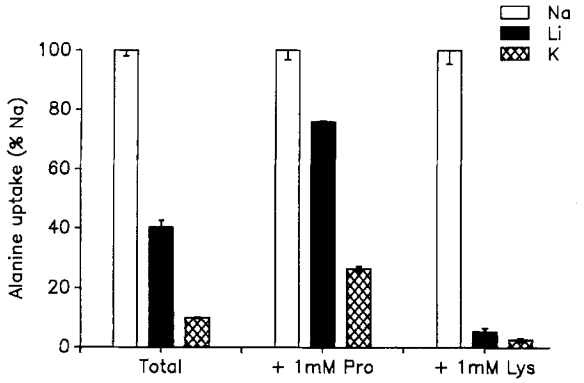

Fig. 3. Cation selectivity of alanine transport pathways. Resuspension buffers were the same as in Fig. 1. Transport buffers contained either NaCl, LiCl or KCl at 260 mM, with 10 mM HEPES-Tris, pH 7.6 and mannitol to maintain isosmolarity. The transport buffers contained 0.5 μM ^{3}H-alanine. Some of the transport buffers also contained 1 mM unlabeled proline (for measurement of alanine uptake via the AK pathway), or 1 mM lysine (for measurement of alanine uptake via the AP pathway). Ten-second uptakes were measured. Uptakes are expressed as percentages of the uptake seen in the presence of Na^+ for each group. The bars represent the means ± SEM of triplicate determinations from a single membrane preparation

Time Course of Lysine and Proline Transport

The results of Figs. 1–3 suggested that lysine and proline are transported by separate pathways in mussel gill BBMV. Therefore, it was decided to examine lysine and proline transport directly as a means to study the two pathways for α-neutral amino acid transport (the AK and AP pathways).

Figure 4 (*A* and *B*) shows the time courses of uptake of lysine and proline in the presence of an inwardly directed sodium gradient (300 mM Na^+ out, 0 in). Intravesicular concentrations of lysine reached a maximum (the "overshoot") at approximately 10 min (Fig. 4*A*), and then decreased to reach an apparent equilibrium concentration by around 6 hr. The overshoot concentration of lysine was 18 ± 3 times that seen at equilibrium (n = 3). The peak of the proline overshoot (Fig. 4*B*) was reached at approximately 30 min, later than that for lysine, and intravesicular proline concentrations approached equilibrium at 6 hr. The later proline overshoot may be related to the relatively low rate of proline uptake in these membranes. The maximal concentration of proline was 5 ± 1.5 times the equilibrium concentration (n = 3). In the presence of millimolar lysine or proline (Fig. 4*A*,*B*), the overshoots were abolished and the remaining uptake into the vesicles probably represented diffusion. As seen previously for alanine (Pajor & Wright, 1987), concentrative uptake of lysine was also seen when Na^+ was replaced by inwardly directed gradients of Li^+ and K^+ (*results not shown*).

Inhibition of Lysine and Proline Uptake by Amino Acids

Figure 5*A* and *B* illustrates the effects of amino acids on the uptake of lysine and proline. The uptake of 0.5 μM ^{3}H-lysine was inhibited by more than 80% by 100 μM concentrations of arginine, lysine, leucine, histidine and alanine. Glycine was an intermediate inhibitor of lysine transport (approximately 40% inhibition), while aspartate, α-(methylamino)isobutyric acid (Me-AIB), and taurine inhibited the transport of lysine by less than 20%. There was no inhibition of lysine transport by proline, or by glucose (*not shown*). In a single experiment, 1 mM lysine or alanine inhibited lysine transport by more than 99%, while 1 mM proline inhibited lysine transport by only 15%.

The most effective inhibitors (>80% inhibition) of proline transport (using 0.5 μM proline and 100 μM inhibitor), were proline, leucine, Me-AIB, and alanine (Fig. 5*B*). Glycine inhibited proline transport by about 60%. There was approximately 30% inhibition of proline transport by histidine and aspartate, approximately 25% inhibition by lysine, taurine, and glucose (*not shown*), and little effect of arginine. In two single experiments, 1 mM lysine inhibited proline transport by only 7%, while 1 mM concentrations of alanine or proline inhibited proline transport by 100 and 90%, respectively.

Kinetics of Lysine and Proline Uptake

Figure 6*A* and *B* shows data from representative experiments examining the kinetics of lysine and proline uptake. In both cases, the kinetics could be described by the sum of a saturable Michaelis-Menten component and a linear diffusive component. Again, it should be noted that the apparent diffusive component could represent a carrier-mediated pathway of low substrate affinity. In experiments with membranes from three separate preparations, the J_{max} for lysine transport was 551 ± 78 pmol/mg-min, with an apparent K_t of 5 ± 1 μM, and P of 11 ± 5 nl/mg-sec. In three experiments with proline, the J_{max} was 184 ± 38 pmol/mg-min, with an apparent K_t of 4 ± 1 μM, and a P of 28 ± 26 nl/mg-sec. These kinetic parameters should be considered estimates since it is likely that there is some overlap in the substrate specificities of the two transport pathways, particularly at high substrate concentrations. The kinetic plots (Fig. 6) probably include low affinity path-

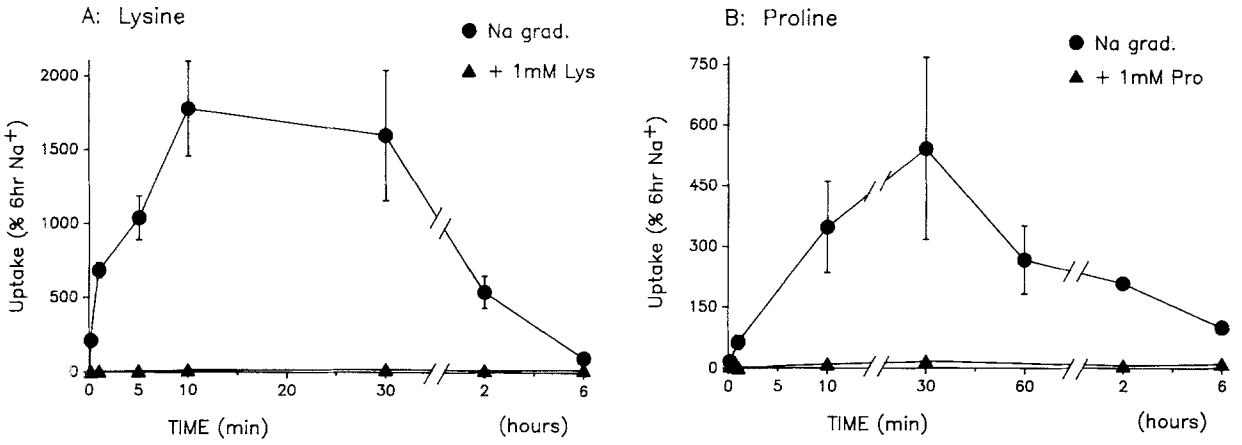

Fig. 4. Time courses of lysine and proline uptake in BBMV. Resuspension buffers were the same as in Fig. 1. Transport buffers contained 0.5 μM ^{3}H-lysine (*A*) or ^{3}H-proline (*B*), plus 300 mM NaCl, 10 mM HEPES-Tris, pH 7.6. Uptake of substrate was measured over 6 hr in the presence and absence of 1 mM substrate. The points represents means ± SEM from experiments with three separate membrane preparations

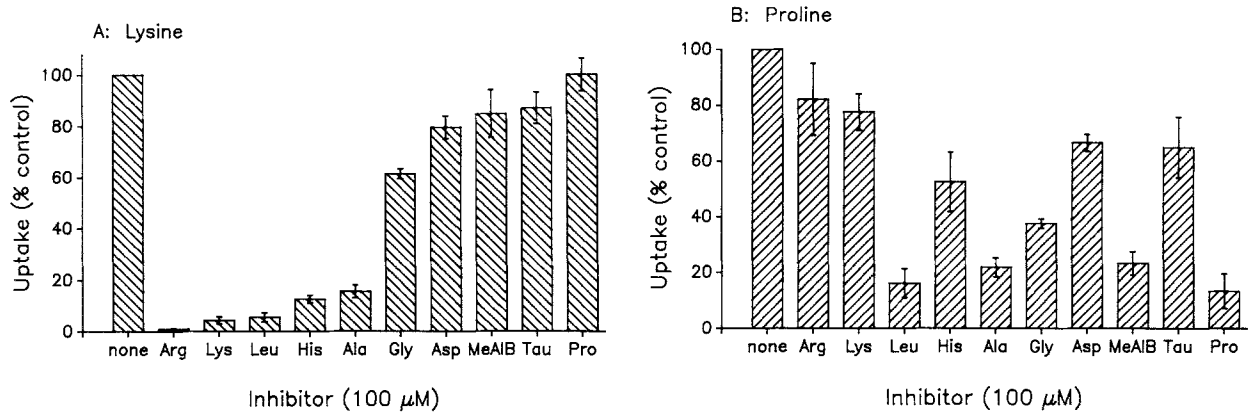

Fig. 5. Inhibition of the initial rate (10 sec) of lysine and proline uptake by amino acids. Resuspension buffers were the same as in Fig. 1. Transport buffers contained 0.5 μM ^{3}H-lysine (*A*) or ^{3}H-proline (*B*), plus 500 mM NaCl, 10 mM HEPES-Tris, pH 7.6, and 100 μM amino acids (*see* figure). The bars represent means ± SEM of experiments with three separate membrane preparations

ways, which were not resolved over the range of concentrations used, for the uptake of lysine via the AP pathway and for the uptake of proline via the AK pathway.

Cation Selectivity of Lysine and Proline Uptake

A more detailed examination of the cation selectivity of the two α-neutral amino acid transport pathways in these membranes was made. As was the case for alanine uptake by the AP pathway (Fig. 3), the uptake of proline was dependent on the presence of Na^+ (Fig. 7*B*). The uptake of proline was inhibited by more than 98% when Na^+ was replaced by Li^+, K^+, choline$^+$, N-methyl-D-glucamine$^+$ (NMG), or sorbitol (Fig. 7*B*).

Figure 7*A* shows the effect of Na^+ replacement on the initial rate of lysine uptake in mussel BBMV. The results resembled earlier observations (*see* Fig. 3) and suggest that the AK transport pathway does not have a strict requirement for Na^+. The replacement of Na^+ by a battery of monovalent cations reduced the uptake of lysine by only 5 to 71% (Fig. 7*A*). More than 99% of lysine uptake, regardless of the cation used, was inhibited by 1 mM lysine, indicating the presence of either a carrier-mediated transport mechanism or specific binding.

Time courses of lysine uptake in the presence of inwardly-directed gradients of Na^+, Li^+, and K^+, as indicated above, showed overshoots in intravesicu-

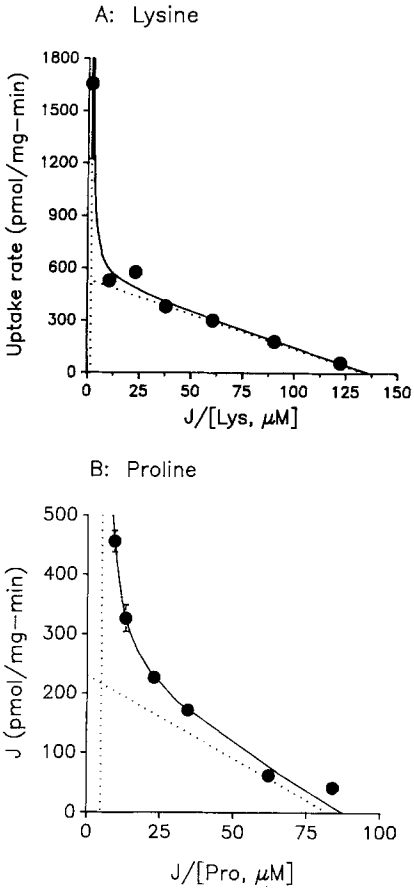

Fig. 6. Kinetics of lysine and proline uptake. Woolf-Augustinsson-Hofstee plots of J, the initial rate (5 sec) of lysine (*A*) and proline (*B*) uptake at increasing substrate concentrations. Resuspension buffers were the same as in Fig. 1. Transport buffers contained 0.5 to 100 μM ^{3}H-lysine or ^{3}H-proline, plus 500 mM NaCl, 10 mM HEPES-Tris, pH 7.6. The points represent means ± SEM (many of the error bars are smaller than the symbols) of triplicate determinations with a single membrane preparation. The solid curves represent total uptake, the dotted vertical lines represent the apparent diffusive portion of this uptake, and the dotted lines with the negative slopes represent the saturable portions of uptake. (*A*) Lysine kinetics: J_{max} 534 pmol/mg-min, K_t 4 μM, P 28 nl/mg-sec. (*B*) Proline kinetics: J_{max} 230 pmol/mg-min, K_t 3 μM, P 80 nl/mg-sec

lar lysine concentration and subsequent reduction in lysine concentrations to reach equilibrium. The apparent equilibrium volumes noted for lysine in Na^+ (11.5 ± 1.3 μl/mg protein (n = 4)), Li^+ (9.4 μl/mg), and K^+ (7.3 μl/mg) were very similar to those previously seen with glucose in these membranes (10.4 ± 1.4 μl/mg (n = 8); Pajor, Moon & Wright, 1989). However, when time courses of lysine uptake (*not shown*) were conducted in the presence of choline, NMG^+, or sorbitol, the apparent equilibrium volumes were very high (choline, 29.3; NMG, 113.9; sorbitol, 163.5 and 221.8 μl/mg), indicating that under these conditions a component of total lysine uptake may have involved binding to the vesicles.

Inhibition by Harmaline

Figure 8*A* and *B* shows Dixon plots of lysine inhibition by harmaline in buffers containing either Na^+ or K^+. In rabbit renal BBMV, it is thought that harmaline inhibits Na^+-dependent alanine and glucose transport by competing with Na^+ at the Na^+-binding site (Aronson & Bounds, 1980). In mussel BBMV (Fig. 8*A*,*B*), harmaline inhibited the transport of lysine in either Na^+ or K^+, and the intersection of the lines in the Dixon plots was consistent with a competitive interaction between harmaline and Na^+ or K^+ (Segel, 1975). The K_i for harmaline inhibition of lysine in the presence of Na^+ was 0.4 mM; in the presence of K^+ the K_i was 1 mM.

Na^+ Activation

Representative experiments of the activation of lysine and proline uptake by increasing concentrations of Na^+ are shown in Fig. 9*A* and *B*. In three experiments that were done at Na^+ concentrations up to 500 mM, the relationship between Na^+ concentration and the rate of lysine or proline uptake was greater than first-order. Since the kinetics of this activation appeared sigmoid rather than hyperbolic, these data indicate that more than one Na^+ is involved in the transport of lysine and proline. The uptake of lysine and proline did not approach saturation, even at 500 mM Na^+, thereby precluding a kinetic analysis of these data. However, this lack of saturation at high Na^+ concentrations suggests that the AK and AP pathways have relatively low affinities for Na^+.

Membrane Potential

The cotransport of proline (a zwitterion with a net neutral charge at pH 7.6) with Na^+, or the transport of lysine (predominantly a cation at pH 7.6) with or without Na^+, should depolarize the vesicles, bringing net positive charge into the vesicles. It was therefore decided to examine the effects of membrane potential on the transport of lysine and proline in BBMV.

The membrane potential was made inside-negative by preloading the vesicles with K^+ and then allowing the K^+ to diffuse out of the vesicles upon the addition of valinomycin. The effects of an inside-negative membrane potential on the transport of glucose, lysine and proline are shown in Fig. 10*A*–*C*. The experiments with lysine and proline

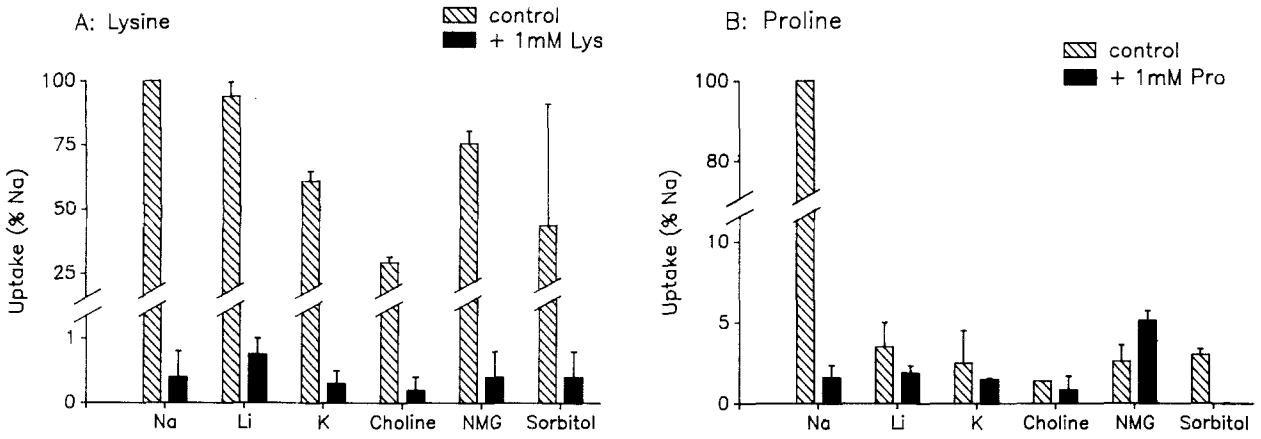

Fig. 7. The effects of cations on lysine and proline uptake. Resuspension buffers were the same as in Fig. 1. Ten-second uptakes of 0.5 μM ^{3}H-lysine (*A*) or ^{3}H-proline (*B*) were measured in transport buffers containing 500 mM NaCl, LiCl, KCl, CholineCl, N-methyl-D-glucamineCl (NMG) or 1 M sorbitol, buffered to pH 7.6 with 10 mM HEPES-Tris. Transport was measured in the absence and presence of 1 mM lysine or proline. The data are expressed as a percentage of the uptake seen in NaCl (without millimolar lysine or proline). The bars represent means ± SEM of experiments with three separate membrane preparations (n = 5 for lysine in Na^+, Li^+, K^+)

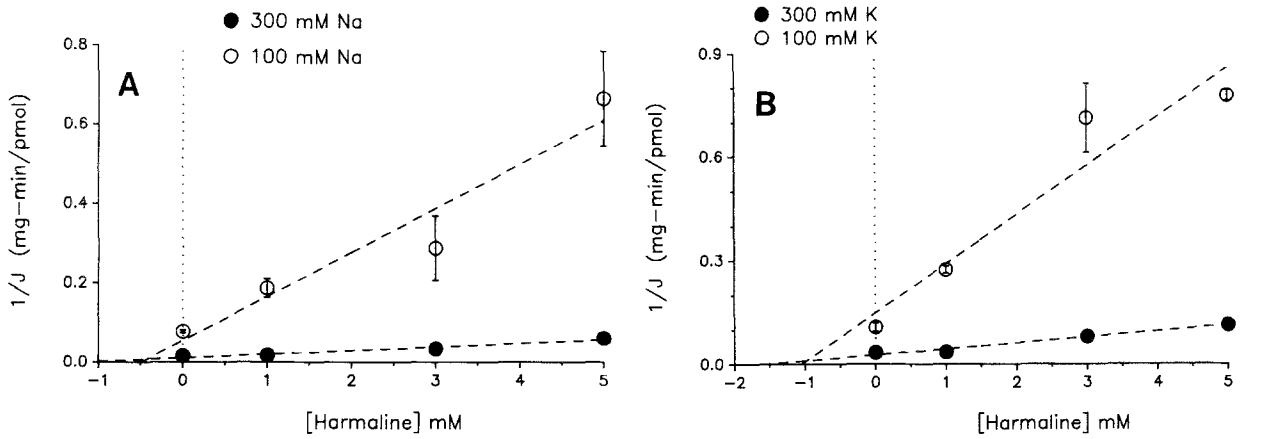

Fig. 8. Harmaline inhibition of lysine uptake. Ten-second uptakes (J) of 0.5 μM ^{3}H-lysine were measured in 300 and 100 mM NaCl (*A*) or in KCl (*B*), with 10 mM HEPES-Tris, pH 7.6 and mannitol to maintain isosmolarity. The transport buffers contained up to 5 mM harmaline. The resuspension buffers were the same as in Fig. 1. The points represent means ± SEM of triplicate determinations from a single membrane preparation

were done in parallel with glucose, since a previous study established that these conditions stimulated both the initial rate and height of the overshoot in glucose transport (Pajor et al., 1989). This result was verified here (Fig. 10*A*). In three separate experiments, there was no effect of an inside-negative membrane potential on the initial rate of lysine transport, though there was an increase (1.4- ± 0.1-fold) in the height of the overshoot. In contrast, the initial rate of proline transport was stimulated (3.4 ± 1.6 times, n = 3) under these conditions, though there was no effect on the height of the overshoot.

The vesicles were made inside-positive by establishing an inwardly directed K^+ diffusion potential with the ionophore valinomycin. Figure 11*A–C* shows that the production of an inside-positive potential inhibited both the initial rates and the overshoots of glucose, lysine and proline transport. Preliminary studies showed that there were no direct inhibitory effects of valinomycin on the transport of glucose, lysine and proline (*results not shown*).

Discussion

The experiments reported here show that there are at least two distinct pathways for the uptake of α-neutral amino acids in the brush border membrane of mussel gills. These pathways were distinguished by their amino acid and cation selectivities.

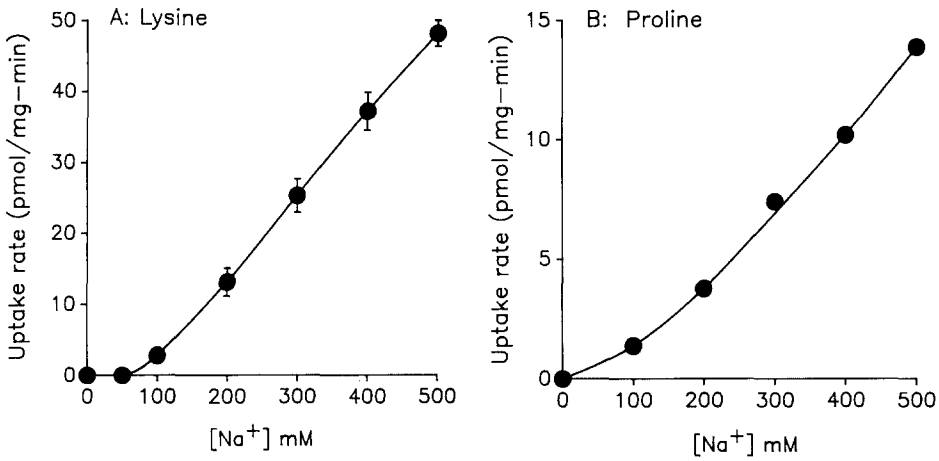

Fig. 9. Na^+ activation of transport. Ten-second uptakes of 0.5 μM ^{3}H-lysine (*A*) or ^{3}H-proline (*B*) were measured in up to 500 mM NaCl (the NaCl was replaced by cholineCl), 10 mM HEPES-Tris, pH 7.6. The points were corrected for uptake in 0 Na^+. Resuspension buffers were the same as in Fig. 1. The points represent means ± SEM (many of the error bars are smaller than the symbols) of triplicate determinations with a single membrane preparation

The alanine-lysine (AK) pathway, which had a high affinity for the neutral amino acid, alanine, and the cationic amino acid, lysine, supported approximately 70% of the total alanine transport capacity under the conditions used in these experiments, and was the major route for alanine uptake in mussel BBMV. There was strong interaction between this pathway and other cationic amino acids and nonpolar α-neutral amino acids. The second route for alanine transport in these membranes, supporting approximately 30% of the total alanine transport capacity, was the alanine-proline (AP) pathway, which showed overlap with the AK pathway in its interaction with the nonpolar amino acids, alanine and leucine. Unlike the AK pathway, the AP pathway transported the imino acid proline, and was strongly inhibited by α-(methylamino)isobutyric acid (Me-AIB). Although the kinetics of alanine transport suggested that the affinity of the AP pathway for alanine is comparatively low, the high apparent K_t for alanine (around 20 μM) may have been a consequence of competitive inhibition by 1 mM lysine at the AP carrier. The high degree of inhibition of proline transport by alanine also supports the idea that the K_t for alanine transport through the AP pathway is probably lower than the apparent K_t obtained in the presence of lysine.

There appeared to be some resemblance between the effects of amino acid inhibitors on the two transport pathways reported here and two of the amino acid transporting pathways described in rabbit kidney brush border vesicles by Mircheff et al. (1982). Like the AK transporter of mussel BBMV, the renal BBMV "System 4" transports lysine and is inhibited by alanine, but has no interaction with proline or Me-AIB. The renal BBMV "System 6" resembles the AP transport pathway of mussel BBMV since System 6 transports proline and Me-AIB, is inhibited by alanine, but is insensitive to lysine. Neither System 4 nor System 6 is affected by the β-amino acid, β-alanine. Similarly, there was little or no effect of the β-amino acid, taurine, on the transport of lysine and proline in mussel BBMV. However, unlike the rabbit Systems 4 and 6, alanine was transported by the two mussel BBMV pathways. It was interesting that Me-AIB interacted here with the transport of proline. The rabbit intestinal IMINO transporter (Stevens & Wright, 1985) is inhibited by both proline and Me-AIB, although alanine does not inhibit this transporter. The two neutral amino acid transport pathways in mussel gills also resembled, in some respects, some of the classical amino acid transporters (Christensen, 1984), such as System A (which transports neutral amino acids and MeAIB, but not proline) and System y^+ (which transports cationic amino acids without Na^+, but may cotransport Na^+ and neutral amino acids).

In addition to their differences in substrate selectivity, the two transport pathways in mussel BBMV had distinct cation selectivities. The low specificity for Na^+ previously observed with alanine transport (Fig. 7; Pajor & Wright, 1987) was a feature of the AK pathway, the major route of alanine transport in these vesicles. In contrast, the AP pathway appeared to have a strict requirement for Na^+.

The cotransport of cations such as Li^+ and K^+, in addition to Na^+, with amino acids has been reported for other systems. Particularly well documented is the cation selectivity of amino acid trans-

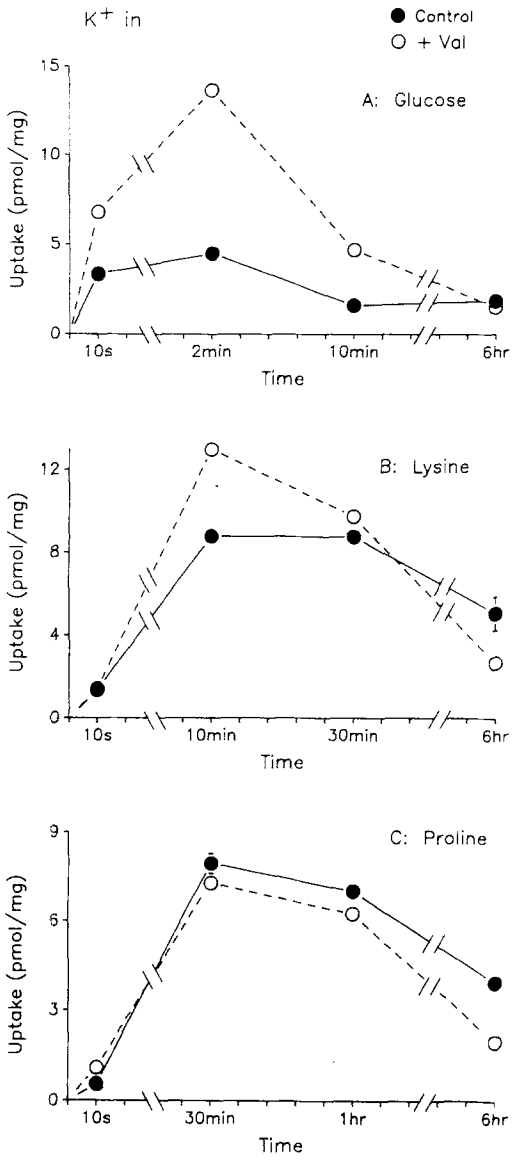

Fig. 10. The effects of a negative-inside membrane potential on time courses of glucose, lysine and proline uptake. Vesicles were preloaded with 300 mM KCl, 10 mM HEPES-Tris, pH 7.6. Transport buffers contained 0.5 μM ^{3}H-glucose (*A*), ^{3}H-lysine (*B*), or ^{3}H-proline (*C*) in 300 mM NaCl, 10 mM HEPES-Tris, pH 7.6, and either ethanol (to 1% total volume, controls) or valinomycin (25 μg/ml final concentration) in ethanol. The three experiments were done with vesicles from the same membrane preparation, and the points represent means ± SEM of duplicate determinations

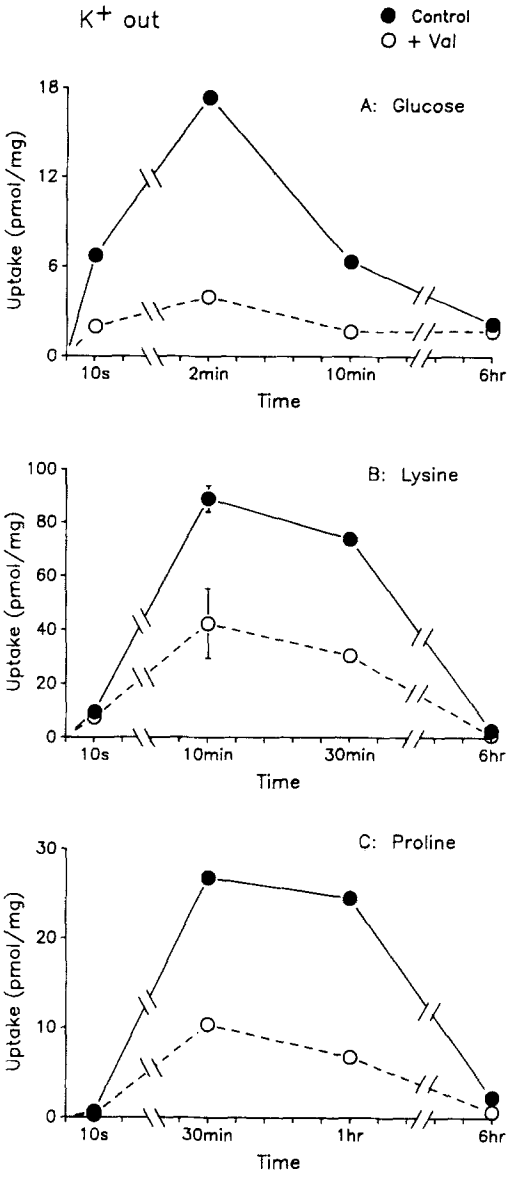

Fig. 11. The effects of a positive-inside membrane potential on time courses of glucose, lysine and proline uptake. Vesicles were preloaded with 600 mM mannitol, 10 mM HEPES-Tris, pH 7.6. Transport buffers contained 0.5 μM ^{3}H-glucose (*A*), ^{3}H-lysine (*B*), or ^{3}H-proline (*C*) in 200 mM NaCl, 100 mM KCl, 10 mM HEPES-Tris, pH 7.6, and either ethanol (to 1% total volume, controls) or valinomycin (25 μg/ml final concentration) in ethanol. The three experiments were done with vesicles from the same membrane preparation, and the points represent means ± SEM of duplicate determinations

port in larval insect midgut. In this system, the transport of alanine supported by K^+ is almost as high as that supported by Na^+ (Hanozet et al., 1984); and the rate of lysine transport is higher in K^+ than in Na^+ (Giordana et al., 1985; Wolfersberger et al., 1987). For example, the rate of Na^+-supported lysine transport in BBMV from the lepidopteran, *Philosomia cynthia*, midgut is 67% of that seen in K^+ (Giordana et al., 1985). The results of a recent study suggest that this phenomenon may exist in another marine invertebrate, the sea anemone, *Anemonia sulcata* (Buck & Schlichter, 1987). These authors reported "overshoots" for leucine transport in sea anemone BBMV in the presence of

inwardly directed KCl gradients. These overshoots were explained as effects of Cl^-. However, Schlichter et al. (1986) reported differences in rates of leucine transport in sea anemone BBMV in the presence of several Cl^- salts (Na^+, Li^+, K^+, and choline$^+$). These data imply that, while Cl^- may be involved, Li^+ and K^+ gradients may also support active transport of leucine in sea anemone BBMV, with the apparent order of cation preference being $Na^+ > K^+ > Li^+$.

The consequences, if any, of the low cation selectivity of the AK carrier in marine mussel gills are presently unknown. Unlike the conditions in the larval insect midgut (*see* Giordana, Sacchi & Hanozet, 1982), the K^+ gradient in mussel gill cells is outwardly directed (approximately 10-fold), and Li^+ is a trace element in seawater. The predominant monovalent cation in seawater is Na^+, and there is approximately a 40-fold inwardly-directed concentration gradient of Na^+ across the gill brush border membrane (425 mM Na^+ out, ≈11 mM Na^+ in; *see* Wright, Moon & Silva, 1989). Furthermore, the membrane potential of gill cells is approximately −60 mV (Murakami & Takahashi, 1975), resulting in a large inwardly directed electrochemical gradient for Na^+ and a negligible electrochemical gradient for K^+. It is likely, therefore, that this transporter functions essentially as a Na^+-amino acid cotransporter with a low affinity for Na^+.

If the Na^+ electrochemical gradient is the sole source of energy for the transport of amino acids in mussel gills, it has been estimated that a coupling of three Na^+ would be adequate to drive uphill amino acid transport against a millionfold gradient (Manahan et al., 1983). Studies with intact mussel gills indicate that at least three Na^+ ions are involved in the transport of alanine and taurine (Wright, 1987). Furthermore, the results of the Na^+-activation experiments in gill BBMV (Fig. 9) suggested that multiple Na^+ ions are involved in the transport of lysine and proline. The data did not lend themselves to Hill analysis, so we have limited our conclusions to saying that more than one Na^+ is involved with the transport of each amino acid.

The neutral amino acids, alanine and glycine, constitute approximately 10% of the gill intracellular amino acid pool (Wright et al., 1987), or a total concentration of about 13 mM, whereas seawater concentrations of total α-neutral amino acids are submicromolar, with estimates ranging between 0.1 and 0.2 μM (Henrichs & Williams, 1985). At the extreme, Manahan et al. (1983) have observed net uptake of alanine in *Mytilus edulis* from concentrations as low as 10 nM, i.e., against a concentration gradient greater than 10^6-fold. The transmembrane proline gradient is probably also large; the intracellular proline pool is at least 1–2 mM (based on estimates by Kluytmans et al., 1980; Strange & Crowe, 1979), while seawater proline concentrations are probably also submicromolar. In contrast, the transmembrane cationic amino acid gradient is likely to be much smaller than that of α-neutral amino acids or proline. Seawater concentrations of total cationic amino acids are around 0.2 μM (Braven, Evens & Butler, 1984; Siebers & Winkler, 1984), but free cationic amino acids are not concentrated in the tissues to any great extent (Wright et al., 1987). Nevertheless, the coupling of more than one Na^+ ion to the transport of amino acids across the apical membrane of integumental cells is probably requisite for the concentrative transport of these substrates.

The influence of membrane potential on the transport of lysine and proline was not as striking as its effect on glucose transport in these membranes (Figs. 10, 11). Both the initial rate and "overshoot" of glucose transport were clearly stimulated by an inside-negative membrane potential and inhibited by an inside-positive potential. In the case of lysine and proline, there were different effects of membrane potential on "overshoots" and initial rates (Figs. 10, 11). This implies that there are more complex (or, at least, different) interactions between lysine or proline transport and membrane potential, and further study will be needed to clarify them. However, the effects of membrane potential on lysine and proline transport were consistent with an electrogenic mechanism of transport (*see* Turner, 1981), thereby supporting the hypothesis that the −60 mV potential difference of the gill apical membrane (Murakami & Takahashi, 1975) can serve as a driving force for transport of amino acids in mussel gills.

In summary, mussel gill brush border membrane vesicles were found to contain at least two pathways for the transport of alanine. One of these, the AK pathway, had a high affinity for α-neutral amino acids, such as alanine, and for cationic amino acids, such as lysine. The other pathway, the AP pathway, also transported α-neutral amino acids, such as alanine, but showed a high affinity for the transport of proline and was inhibited by Me-AIB. The AK pathway had a low Na^+ specificity, while the AP pathway had a strict requirement for Na^+. Multiple Na^+ ions and membrane potential were involved in providing energy for concentrative transport of these amino acids in BBMV, supporting the hypothesis that these serve as driving forces for net amino acid transport in the intact animal against concentration gradients that can exceed 10^6 to 1.

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