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Alanine and Taurine Transport by the Gill Epithelium of a Marine Bivalve: Effect of Sodium on Influx

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Summary. Marine mussels can accumulate amino acids from seawater into the epithelial cells of the gill against chemical gradients in excess of 5×10^6 to 1. Uptake of both alanine and taurine into gill tissue isolated from *Mytilus californianus* was found to be dependent upon Na^+ in the external solution. Uptake of these amino acids was described by Michaelis-Menten kinetics, and a reduction in external [Na^+] (from 425 to 213 mM) increased the apparent Michaelis constants (alanine, from 8 to 17 μM; taurine, from 4 to 39 μM) without a significant influence on the J_{max}'s of these processes. Five mM harmaline, an inhibitor of Na-cotransport processes in many systems, reduced both alanine and taurine uptake by more than 95%; this inhibition appeared to be competitive in nature, with an apparent K_i of 43 μM for the interaction with alanine uptake. Increasing the external [Na^+] from 0 to 510 mM produced a sigmoid activation of alanine and taurine uptake with K_{Na}'s of approximately 325 mM. The apparent Hill coefficients for this activation were 7.3 and 7.4 for alanine and taurine, respectively. These data are consistent with uptake mechanisms which require comparatively high concentrations of Na^+ to activate transport, and which couple several Na^+ ions to the transport of each amino acid. These characteristics, in conjunction with the previously demonstrated low passive permeability of the apical membrane to amino acids, result in systems capable of i) accumulating amino acids from seawater to help meet the nutritional needs of this animal, and ii) maintaining the high intracellular amino-acid concentrations associated with volume regulation in the gill.

Key Words epithelia · integument · coupled transport · amino-acid transport · taurine · alanine · marine bivalve · *Mytilus californianus*

Introduction

The abilty of marine bivalves to accumulate amino acids from dilute external solution has received widespread attention from physiological ecologists, with the principal focus being on the role of such uptake in whole animal nutrition [35] and osmoregulation [9]. The primary site of uptake of small organic molecules is the gill [16, 24], which has several transport pathways for amino acids. The capacity and apparent affinity for substrate of these processes are such that they could play a significant role in the nutritional physiology of these animals [35].

The energetics of these "integumental" transporters is particularly intriguing. The cells of the gill, as well as those of other organs in the mussel, are characterized by extremely large concentrations of several amino acids which are believed to be important in isosmotic volume regulation [13]. The largest constituent of the free amino acid (FAA) pool of the gill is taurine, with concentrations on the order of 50 to 100 μg/g wet tissue weight [39, 42]. The taurine transporter (i.e., β-zwitterionic amino acids) of the gill is capable of a net accumulation of this substrate from ambient levels in seawater of <20 nM into an intracellular pool of $\simeq 0.1$ M [39]; in other words, the steady-state electrochemical taurine gradient across the brush border of gill cells against which net transport can occur is $\simeq 5 \times 10^6$ to 1.

Integumental amino acid transport in gill tissue has been shown to be sensitive to the concentration of Na^+ in seawater [2, 30, 36, 41]. This observation has led to the suggestion (e.g., ref. 25) that the energy for concentrative uptake of amino acids in the gill is derived from the inwardly directed electrochemical gradient for Na^+ via a coupling between the fluxes of Na^+ and amino acid. The present study was designed to examine the influence of Na^+ on the kinetics of transport of two amino acids transported by separate processes in the gill, alanine and taurine. The uptake of each was found to be dependent on the presence of Na^+ in the ambient medium. Furthermore, the kinetics of Na^+'s interaction with the transporters supported the idea that as many as three Na^+ ions may be coupled to the flux of each substrate molecule, offering a potential explanation for the observed magnitude of the amino-acid gradients sustained by the gill. Finally, the passive and

active permeability characteristics of the gill were considered in terms of the gills' ability to maintain FAA pools used for volume regulation.

Materials and Methods

Animals

Specimens of the California coastal mussel (*Mytilus californianus*) were purchased from the Bodega Marine Biological Laboratory, and were kept in a refrigerated (13°C) aquarium containing filtered artificial seawater (Instant Ocean). Animals were routinely used within 6 weeks of collection.

Test Solutions

The seawater formulation described by Cavanaugh [5] was used for all experiments in which a "normal" Na^+ concentration was desired. The composition of this seawater (henceforth referred to as Na-ASW) was (in mM): NaCl 423, KCl 9, $CaCl_2$ 9, $MgCl_2$ 23, $MgSO_4$ 26, $NaHCO_3$ 2. When Na^+ was replaced by other monovalent cations, NaCl was replaced by the appropriate chloride salt, and $NaHCO_3$ was replaced with $KHCO_3$.

Transport Measurements

Uptake of amino acids into isolated gill tissue used the following protocol. Individual demibranchs (2 per gill, 4 per animal) were removed and preincubated in Na-ASW. The demibranchs were cut into two or three pieces, and a piece of 000 silk suture tied around an end of each. The tissue was preincubated at room temperature (21 to 24°C) for 30 min. To activate the lateral cilia, tissue was placed in ASW containing 10 μM 5-hydroxytryptamine (5-HT; ref. 1) 10 min prior to beginning the experiments. The subsequent reduction in unstirred layers between adjacent gill filaments reestablishes a pattern of perfusion of gill surfaces that mimics the in vivo situation, thereby stimulating amino-acid uptake [34, 40]. Separate experiments have shown that 5-HT does not have a direct effect on transport in the gill [34]. Measurements of uptake were started by suspending a piece of gill tissue in a 200-ml test solution containing ^{14}C-labeled amino acid, unlabeled amino acid to produce the desired substrate concentration, 10 μM 5-HT, and artificial seawater of the desired composition. The solution was mixed through the gentle action of a magnetic stir bar. Previous studies showed that uptake of amino acids into *M. californianus* gill tissue is linear for at least one hour (e.g., ref. 38). For most of the present studies, 5-min uptakes were used to estimate the initial rate of influx. Uptake was stopped by rapidly removing the tissue from the test solution and suspending it in an ice-cold Na-ASW rinse containing 10 μM 5-HT for 5 min; this procedure is adequate to flush extracellular compartments of adherent labeled substrate [34]. Preliminary experiments revealed that there was no significant loss of accumulated substrate (<1%) from cellular compartments during the 5-min rinse (*unpublished observations*). Following the cold rinse, uniform, 7-mm disks of tissue were cut from the demibranchs using a stainless steel punch; typically 5 to 9 pieces of tissue were cut from a single section of demibranch. Individual disks were placed into scintillation vials, and accumulated radioactivity extracted with 0.7 ml of 0.1 N HNO_3. After at least 2 hr of extraction, 10 ml of scintillation cocktail was added to each vial and radioactivity determined with a Beckman model 3801 liquid scintillation counter. Data were corrected for variable quench using H number analysis.

Uptake was routinely normalized to gill weight to facilitate comparison with other studies. There was no difference in the variance of uptakes by individual tissue disks when normalized to fresh wet *vs.* dry weight ($P > 0.05$; dry weight was 23% of fresh weight). Thus, for purposes of convenience, data were expressed per gram wet weight of gill tissue, and an average value for the wet weight of 7 mm tissue disks was determined from disks cut from 12 different mussels: 7.7 ± 1.62 mg (SD). For individual animals, standard deviations averaged ±21% of the mean (n's ranging from 20 to 62). In four experiments in which the weight of individual tissue disks was recorded, there was no difference in either means or standard deviations noted between uptakes expressed per gram of measured weight *vs.* uptakes expressed as a function of the mean weight of the population of tissue disks from the animal ($P > 0.05$).

Oxygen Consumption

Rates of oxygen utilization (Q_{O_2}) by isolated gill tissue were measured using a Clark-type electrode with tissue held in a water-jacketed (22°C) metabolic chamber.

Calculations

Comparisons made between test groups to gauge the statistical significance of observed differences were based upon Student's t-test; differences at the 0.05 level were considered significant. Kinetic parameters were calculated using a nonlinear regression program for two-parameter equations [11].

Chemicals

^{14}C-taurine (100 to 120 mCi/mmol) and ^{14}C-alanine (135 to 165 mCi/mmol) were purchased from Amersham and ICN, respectively. Harmaline hydrochloride was purchased from Sigma Chemical Corp. All other chemicals were acquired from standard sources and were the highest grade available.

Results

Effect of Replacement of Na^+ on the Transport of Alanine and Taurine

When Na^+ in the ASW was replaced by either Li^+, K^+, or choline$^+$, uptake of both alanine and taurine was reduced by more than 94% (Fig. 1). In three experiments, there was no apparent pattern to the inhibition of uptake produced by replacement of Na^+ with these three ions, and none appeared to be an effective replacement for Na^+ in the uptake process.

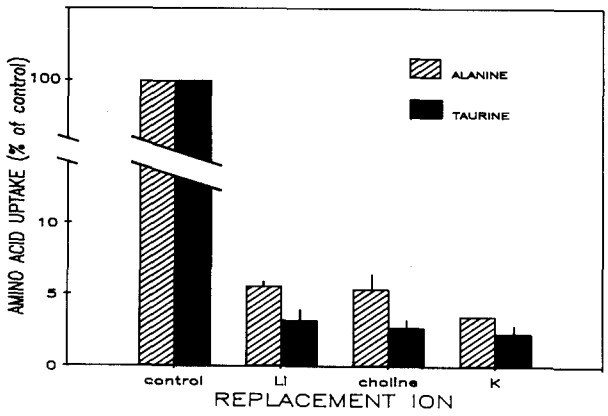

Fig. 1. Effect on alanine and taurine uptake into gill tissue of replacing Na^+ with other cations. Isolated demibranchs were exposed for 5 min to ASW solutions containing 0.5 μM ^{14}C-alanine (hatched bars) or ^{14}C-taurine (solid bars) and either 425 mM Na^+ (control) or ASW in which Na^+ had been replaced with either Li^+, choline$^+$, or K^+. Data is expressed as the percent of uptake noted in the presence of Na^+ (note the break in the units of the ordinate); vertical lines above the bars represent the standard error of the mean of separate experiments with tissue from three different animals. As is the case in all experiments reported here, uptakes for a given test condition in each individual experiment were derived from accumulation of labeled substrate into between 5 and 9 separate pieces of tissue cut from a segment of demibranch

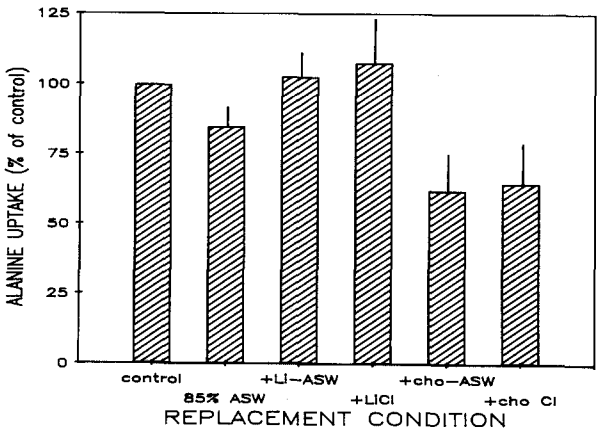

Fig. 2. Inhibitory effects of Li^+ and choline$^+$ on 0.5 μM alanine uptake into gill tissue. The control condition consisted of incubating gills in 85% Na-ASW (diluted with distilled water), with 152 mM sucrose added to achieve the osmolarity of 100% ASW (≈925 mOsm). The control rate of uptake was compared to that measured in gills exposed to i) 85% Na-ASW (i.e., hypoosmotic), or 85% Na-ASW solutions whose osmolarity had been adjusted to normal through the addition of either ii) 15% Li-ASW, iii) 15% choline-ASW, iv) 63 mM LiCl, or v) 63 mM choline chloride (isosmolarity with ASW achieved through the addition of sucrose). Uptakes are expressed as a percentage of the control condition, and the vertical bars represent +1 SE (n = 3)

Li^+ has been shown to be a competitive inhibitor at one or more of the Na^+ binding sites involved in several Na-coupled cotransport processes (e.g., refs. 20, 33). However, rates of alanine uptake were not significantly influenced by the presence of 63 mM Li^+, though exposure of gill tissue to media containing 63 mM choline$^+$ did cause a significant inhibition of uptake (Fig. 2). These observations indicated that Li^+ is not a potent competitive inhibitor of Na^+'s interaction with the transport process. Furthermore, they suggested the advisability of using Li^+ as a routine replacement for Na^+, rather than choline, in subsequent studies of the kinetics of Na^+'s interaction with amino acid transport in the gill.

Exposure of the gill to Na-free media could have had a serious effect on the viability of the tissue, thereby complicating the interpretation of Na-replacement studies. Measurement of O_2 consumption (Q_{O_2}) of isolated gill tissue did reveal that a 15-min exposure to Li-ASW caused a 32% reduction in Q_{O_2} [0.73 ± 0.047 (n = 4) to 0.50 ± 0.008 (n = 3) ml O_2/(g-hr)]. Consequently, it was of interest to determine whether the removal of Na^+ caused a reversible inhibition of amino acid uptake. Gill tissue was exposed for 6 min to a Na-free ASW, after which the tissue was allowed to recover in Na-ASW for increasing periods of time before a 5-min uptake was determined. The results of several experiments in which alanine uptake was measured are shown in Fig. 3. Two observations are worth noting. First, the inhibition caused by removing Na^+ from test solutions was at least partially reversible. For example, though immediately following exposure to Li-ASW alanine uptake in Na-ASW was reduced by 96%, rates returned to the control level within 60 min. A similar approach to control values of uptake occurred when tissue was exposed to K^+ and choline$^+$ (Fig. 3). Two separate experiments revealed a qualitatively similar profile for recovery of taurine uptake rates following complete replacement of Na^+ with Li^+: after 30 min of recovery, uptake was 16% of control; after 60 min, uptake was 123% of control.

The second point is the surprisingly long length of time that it took for uptake to recover. Uptake in Na-free ASW was, as we have seen, reduced by approximately 95%, but the subsequent reexposure to Na-ASW did not produce a dramatic "reactivation" of transport. The final extent of recovery took between 30 and 90 min. Thus, the inhibition of alanine and taurine uptake resulting from removal of Na^+ appears to involve more than the acute effects of Na^+ at the transport sites.

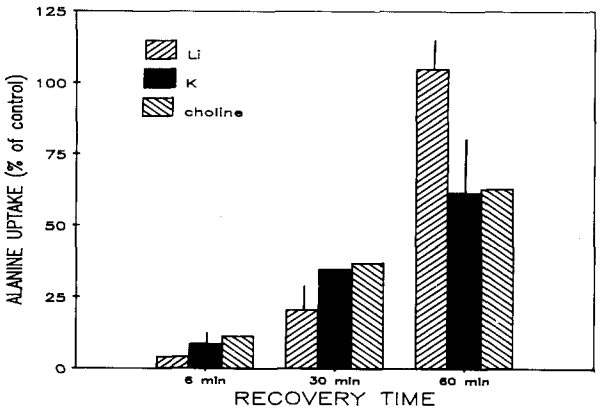

Fig. 3. Recovery of alanine uptake in gill tissue exposed to Na-free ASW. Tissue was exposed for 6 min to Na-free ASW solutions in which Na^+ was replaced with either Li^+, K^+, or choline$^+$. The tissue was then placed into normal Na-ASW for either 6, 30 or 60 min, after which a 5-min uptake of 0.5 μM ^{14}C-alanine was measured. Rates of uptake are expressed as a percentage of the uptake measured in parallel experiments of uptake into gill tissue that had not been exposed to a Na-free ASW. The observations made with Li^+ and K^+ were based on the results of studies with two different animals, and the vertical lines indicate +one-half the range noted in these experiments. The choline results represent observations made with tissue from one mussel

Effect of Na^+ on the Kinetics of Uptake

As shown in Fig. 4*A*, alanine uptake from ASW containing either 425 mM Na^+ (i.e., 100% Na-ASW) or 213 mM Na^+ (i.e., 50% Na-ASW) was adequately described by the Michaelis-Menten equation:

$$J = \frac{J_{max}[A]}{K_t^* + [A]} \quad (1)$$

where J is the rate of uptake from an external alanine concentration of $[A]$, J_{max} is the maximal rate of uptake, and K_t^* is the apparent Michaelis constant. It is worth noting that the K_t^* determined in studies with isolated gill tissue is expected to overestimate the "true" Michaelis constant because the laminar, unidirectional flow of water through the gill results in a systematic reduction of substrate concentration as fluid flows past the transport surfaces; the half-saturation constant of the intact gill is actually about 1.5 to 5 times greater than that of the K_t of the transporter [14, 38]. The J_{max} should not be influenced by this situation [38].

Alanine uptake in both 100 and 50% Na-ASW was clearly saturable with no indication of any parallel, nonsaturable components to the uptake process. At every alanine concentration examined, uptake in 50% Na-ASW was less than that determined in 100% Na-ASW. This reduction appeared to involve an increase in the apparent Michaelis constant for alanine uptake; in four separate experiments the mean K_t^* in 100% Na-ASW was 7.7 ± 0.91 μM, which increased to 17.4 ± 1.77 μM in 50% Na-ASW ($P < 0.01$). The J_{max} for alanine uptake was not significantly affected by the reduction in external $[Na^+]$ (J_{max} at 100% Na-ASW = 39 ± 3.7 μmol/(g-hr); J_{max} at 50% Na-ASW = 33 ± 3.1, $P > 0.05$), though in every experiment the J_{max} measured under the reduced Na^+ condition was less (≃15%) than the control value. In similar studies on the effect of Na^+ on taurine uptake (Fig. 4*B*), the K_t^* was also influenced to a greater extent by a reduction in ambient Na^+ than was J_{max}. In two experiments at 100% Na-ASW, the mean J_{max} for taurine was 11.4 ± 2.71 μmol/(g-hr) (the error here denotes ±one-half the range of the two experiments), with a K_t^* of 4.0 ± 0.57 μM. Exposure to 50% Na-ASW produced a significant elevation of K_t^* to 39.3 ± 5.17 μM ($P > 0.05$; $n = 3$), without a reduction of J_{max} (8.2 ± 2.75 μmol/(g-hr); $P > 0.05$; $n = 3$). Thus, while effects of Na^+ on the maximal rate of integumental uptake processes should not be dismissed, it seems clear that the primary effect of external Na^+ was on the apparent affinity of the transporters for alanine and taurine.

One caveat should be emphasized at this point. Separate studies on substrate inhibition suggest that, while taurine uptake in the gill appears to involve a single carrier-mediated pathway [36, 38], alanine transport involves (at least) two separate, Na-dependent transport processes [36]. Each has a broad, overlapping specificity for neutral, zwitterionic amino acids, though one shows a selectivity for proline while the other shows selectivity for lysine. The present results, however, indicate that these separate pathways have similar kinetic characteristics, i.e., transport could not be resolved into two separate components (e.g., high affinity *vs.* low affinity). Therefore I elected to treat alanine uptake as if it occurred via a single process, and subsequent interpretation of the kinetics of alanine uptake should be considered in light of that simplification.

Effect of Harmaline on the Uptake of Alanine and Taurine

The plant alkaloid, harmaline, blocks the Na-dependent transport of a variety of compounds, apparently acting as a competitive inhibitor at Na-binding sites [4]. The drug did inhibit both alanine and taurine uptake by the gill in a concentration-dependent manner (Fig. 5), with 5 mM harmaline reducing transport by more than 98%. The kinetics of the

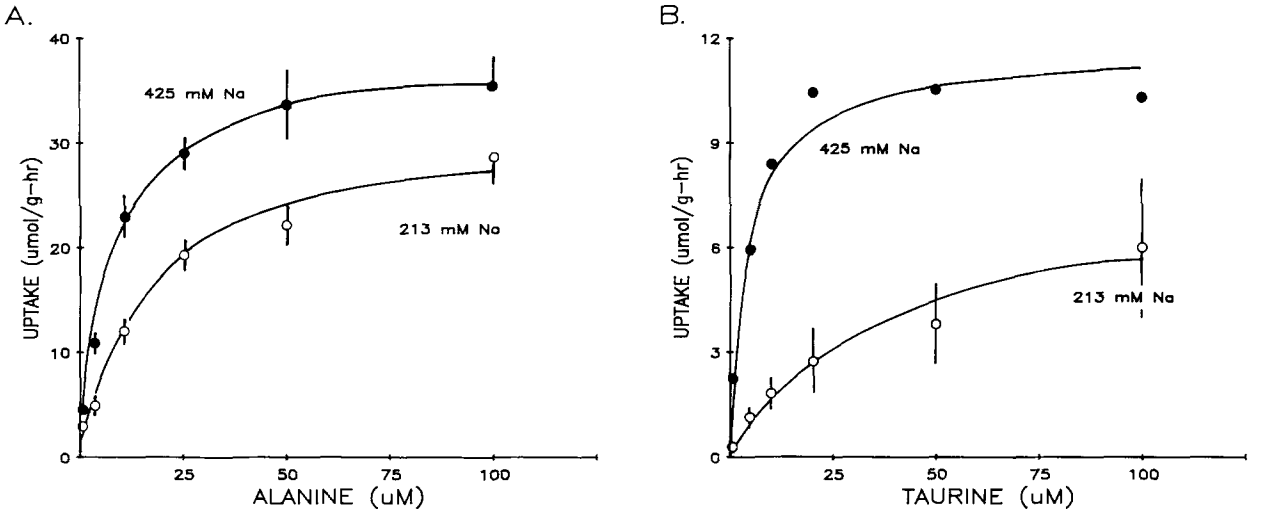

Fig. 4. Effect of Na^+ on the concentration-dependency of (*A*) alanine and (*B*) taurine uptake into gill tissue. (*A*) Presented here are averages from experiments with tissue from four different animals; tissue from each animal was used to study uptake under both experimental conditions, i.e., alanine uptake in the presence of 100% Na-ASW (425 mM Na^+) and 50% Na-ASW (213 mM Na^+; Na replaced with Li^+). Errors are ± 1 SE. The lines were calculated from kinetic constants determined using a nonlinear regression routine for small computers [11]: for 425 mM Na, J_{max} = 38.7 ± 0.81 μmol/(g-hr), K_t^* = 7.8 ± 0.65 μM; for 213 mM Na, J_{max} = 32.2 ± 1.86 μmol/(g-hr), K_t^* = 16.6 ± 3.03 μM. (*B*) The data for taurine uptake in 100% Na-ASW are from two experiments with different mussels, while that for uptake in 50% Na-ASW are from three experiments; in one experiment, tissue from the same animal was used to examine uptake from both 50 and 100% Na-ASW. The lines were generated using the following kinetic constants: for 425 mM Na, J_{max} = 14.1 ± 0.53 μmol/(g-hr), K_t^* = 3.5 ± 0.64 μM; for 213 mM Na, J_{max} = 13.6 ± 0.98 μmol/(g-hr), K_t^* = 45.7 ± 7.24 μM

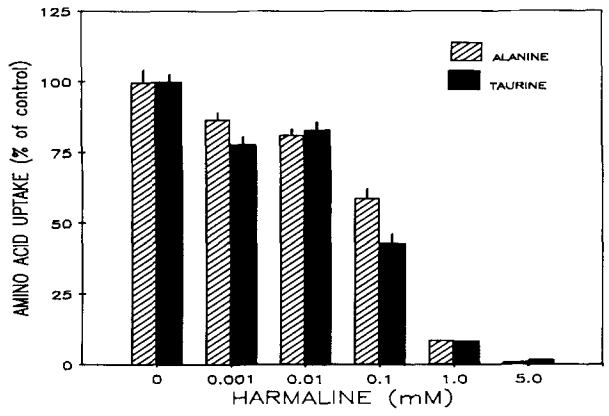

Fig. 5. Effect of harmaline on the uptake of alanine and taurine into gill tissue. Uptakes were measured in 5-min exposures of gill to Na-ASW containing 0.5 μM ^{14}C-alanine and increasing concentrations of harmaline. Data for uptake of alanine were from an experiment with tissue from one mussel; rates are expressed as the percentage of the control uptake with the vertical lines indicating +1 SE (n = 6 pieces of tissue at each experimental condition). Data for uptake of taurine were from a separate experiment with one mussel (n = 5 pieces of tissue at each experimental condition)

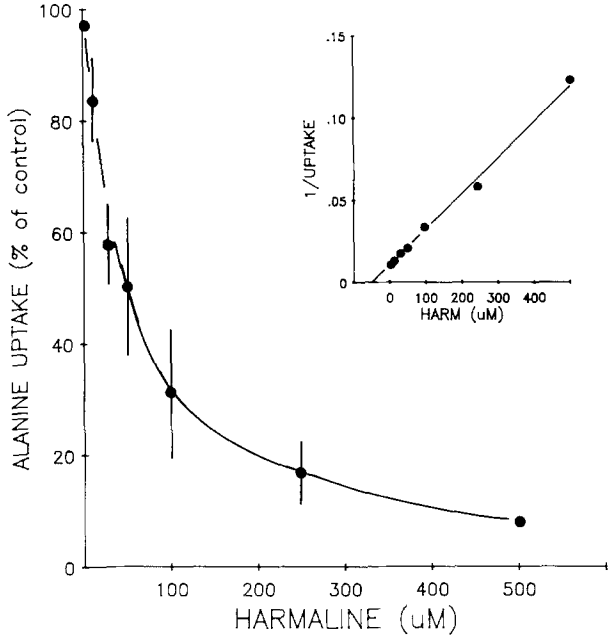

Fig. 6. Kinetics of harmaline inhibition of alanine uptake in gill tissue. Lower graph: Demibranchs were exposed to Na-ASW containing 0.5 μM ^{14}C-alanine and from 1 to 500 μM harmaline. Uptakes are expressed as a percentage of uptake noted in the absence of harmaline. Data points represent the mean of uptakes from separate experiments with three animals; error bars are ± 1 SE. Inset: A Dixon-plot of these data, emphasizing that harmaline inhibition of alanine uptake can be adequately described by the kinetics of competitive inhibition. The apparent K_i for harmaline from this plot was 43 μM

interaction of harmaline with alanine transport are shown in Fig. 6. The hyperbolic nature of this inhibition is supported by the linearity of the Dixon-type plot shown in the inset of Fig. 6. These data suggest that the binding of a single harmaline mole-

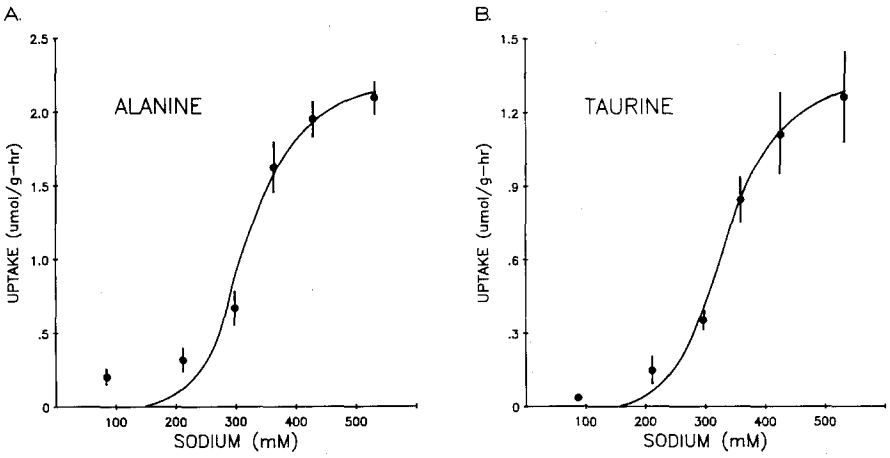

Fig. 7. Effect of increasing Na^+ concentration in ASW on the activation of (*A*) alanine and (*B*) taurine uptake into gill tissue. Demibranchs were incubated for 5 min in ASW containing 0.5 μM ^{14}alanine or ^{14}C-taurine and increasing concentrations of Na^+, with Li^+ used as the replacement ion for Na^+. For the alanine study, each point is the mean uptake from separate experiments with four animals (alanine) ± 1 SE. For taurine, each point is the mean of uptakes measured in two separate experiments ±one-half the range. The lines were fit to the data using a nonlinear regression routine for three parameter equations (T. Reedy, *personal communication*). For alanine, J_{max} was 2.2 μmol/(g-hr), K_{Na} for Na^+ was 321 mM, and the apparent Hill coefficient, n, was 7.3. For taurine, the J_{max} was 1.34 μmol/(g-hr), K_{Na} was 335 mM, and the apparent n was 7.4

cule to an alanine transporter was sufficient to block the uptake process. The apparent K_i for harmaline was 43 μM.

Activation of Alanine and Taurine Transport by Na^+

Uptake of 0.5 μM alanine or taurine was examined as a function of increasing [Na^+] in an effort to gain further information about the interaction between Na^+ and substrate transport in the gill. As shown in Fig. 7, Na^+ activation of alanine and taurine uptake was clearly nonhyperbolic. For purposes of comparison with the results of studies in other tissues, these data were analyzed using the familiar Hill relationship [27]:

$$J = \frac{J_m + [\mathrm{Na}]^n}{K_{\mathrm{Na}} + [\mathrm{Na}]^n} \qquad (2)$$

where J_m is the maximal rate of uptake of the 0.5 μM substrate, K_{Na} is the concentration of Na^+ causing half-maximal uptake, and n is the apparent Hill coefficient. The n values derived from nonlinear regression analyses of the data presented in Fig. 7 were 7.3 and 7.4 for alanine and taurine, respectively. The K_{Na}'s were 321 and 335 mM for alanine and taurine, respectively. The uptakes were not adequately described if n was assumed to be 1, regardless of the values of J_m and K_{Na} used. The comparatively poor fit of the alanine data at low levels of Na^+ (≤212 mM) may be a reflection of differences in the kinetics of Na^+'s interaction with the two (or more) alanine transport pathways in the gill.

The absolute value of these apparent Hill coefficients should be interpreted with caution. First, as pointed out earlier, analysis of kinetic data derived from studies with intact gill tissue is complicated because not all transport sites are exposed to the same concentration of substrate due to the geometry and fluid-flow characteristics of the gill [38]. Second, this experimental preparation does not permit control of factors such as membrane potential which influence transport and which may change as a function of the external ionic composition. Third, conclusions about transport mechanism based upon Hill analysis imply the acceptance of a set of assumptions that are not necessarily warranted without independent information (e.g., high cooperativity between multiple binding sites; ref. 27). Finally, it should be emphasized that Hill coefficients do not indicate the number of Na^+ ions that are coupled to the flux of amino acid. Rather, they are an indication of the (usually minimum) number of ions "involved" with the uptake process. But some of those ions may serve only as kinetic activators of uptake and not be cotransported substrates. Nevertheless, the observations in Fig. 7 indicate that i) more than one Na^+ ion is associated with the transport of

alanine and taurine into gill epithelial cells, and ii) that as many as 3 or 4 Na^+ ions could be coupled to the uptake of each of these substrates.

Discussion

Studies of transport in a wide variety of animal systems have consistently shown that "concentrative" transport of amino acids (i.e., transport against an electrochemical gradient) requires extracellular Na^+. Thus the observed Na-dependence of alanine and taurine transport into surface epithelial cells of *Mytilus* gill was expected. Nevertheless, because of the capacity of the gill to accumulate amino acids against extreme electrochemical gradients, the present results provide some interesting insights into the energetics of secondary active transport processes and the strategies used by cells to maintain large intracellular amino acid concentrations.

The energy for concentrative amino acid transport into many animal cells appears to be derived from the inwardly directed electrochemical gradient for Na^+ through a direct coupling between the fluxes of Na^+ and the amino acid substrate (*see* ref. 8). Though there have been reports of several interesting exceptions to this general statement (e.g., refs. 6, 15, 26), the "Na^+-gradient hypothesis" is accepted as an adequate explanation for most examples of uphill amino acid transport. However, tests of the adequacy of Na^+-electrochemical gradients to account for observed tissue-to-medium (T/M) substrate concentration ratios have been limited by the comparatively low T/M ratios developed by Na^+-coupled transport processes in vertebrate systems, and a subsequent "signal-to-noise" problem. Apparent maximum T/M ratios range from about 70:1 (glucose transport in avian enterocytes; ref. 18) to several thousand to one (taurine transport in ascites cells; ref. 7). Thus it is worthwhile to emphasize that *Mytilus* is capable of a net removal of amino acids from levels in surrounding seawater of less than 20 nM into a free cell pool in the surface epithelium that can be conservatively estimated to be on the order of 0.01 to 0.1 M (i.e., 100 μmol/g wet wt of tissue; ref. 39); i.e., net flux can occur against chemical gradients of $>10^6:1$.

Transport of amino acids against such extreme chemical gradients provides an interesting examination of the adequacy of Na^+ electrochemical gradients to support a secondary active transport. The relationship between the Na^+ electrochemical energy gradient and the energy stored in the gradient for a zwitterionic (i.e., net charge of 0 at pH 7.8 to 8.0) amino acid, A, is given by the following inequality [3]:

Table. Parameters for estimation of stoichiometry of coupling between Na^+ and taurine influxes into *Mytilus californianus* gill

Parameter	Value	Reference
$[A]_i$	100×10^{-3} M	[39]
$[A]_o$	15×10^{-9} M	[39]
$[Na]_i$	17×10^{-3} M	[12]
$[Na]_o$	293×10^{-3} M[a]	
ψ	-0.060 V	[23]
RT/F	2.5×10^{-2} V^{-1}	

[a] Based on an activity coefficient of 0.69 for Na^+ in ASW.

$$\frac{[A]_i}{[A]_o} \leq \left[\frac{[Na^+]_o}{[Na^+]_i} \exp \frac{-\psi F}{RT}\right]^{n_{Na^+}/n_A} \quad (3)$$

where the subscripts i and o refer to the chemical activities of amino acid and Na^+ in cytoplasmic and seawater solutions, respectively; n_{Na^+} and n_A are the numbers of Na^+ ions and alanine molecules co-transported during a single translocation cycle of the transporter; ψ is the electrical potential difference (PD) across the apical membrane of the gill epithelium; F is the Faraday constant; R is the gas constant; and T is the absolute temperature. Most of these parameters have either been measured or can be estimated (*see* the Table). It is worthwhile to note that implicit in the use of Eq. (3) is the simplifying assumption that amino-acid transport in the gill is not influenced by the co- or countertransport of ions other than Na^+.

Na-dependent taurine transport in *Mytilus* gill provides a particularly interesting example for the purposes of this calculation; at $\simeq$100 μmol/g wet wt of gill tissue [39, 42], it is the largest constituent of the free amino acid pool. Of course, this high concentration does not necessarily imply equally high *activity* of taurine in gill cells. However, the well-documented role of taurine as an osmotic effector in cell volume regulation in *Mytilus* (e.g., refs. 13, 21), argues against a major fraction of this material being bound or compartmentalized within gill cells. Thus, until contrary evidence is available, I will assume that integumental transporters can sustain a steady-state intracellular taurine activity of 100 mM. A previous study [39] demonstrated that intact *M. californianus* can accumulate taurine from external concentrations of less than 20 nM, supporting the contention that net taurine flux into gill cells occurs against an electrochemical gradient of $\simeq 5 \times 10^6$.

It is clear from inspection of Eq. (3) that the energy in the Na^+ gradient is not adequate to support observed taurine transport if the coupling between Na^+ and taurine fluxes were 1 to 1. Rearrangement of Eq. (3) permits calculation of the

minimum value for a Na/taurine coupling coefficient that will result in a balance between the energy in the gradients for Na^+ and taurine; based on the information listed in the Table, this value is 2.9, suggesting that at least 3 Na^+ ions must be cotransported with every taurine molecule transported into the gill. Similar calculations for alanine transport are also consistent with a minimum of 3 Na^+ ions being cotransported with this substrate.

The influence of Na^+ on the kinetics of amino-acid transport provides several clues concerning the mechanism(s) by which gill cells maintain large intracellular pools of amino acid. First, the sigmoid nature of the Na-activation of alanine and taurine transport (Fig. 7) supports the predictions just put forward that at least three Na^+ ions must be coupled to the transport of taurine and alanine in the gill if the Na^+ electrochemical gradient is to be an adequate energy source to sustain observed T/M substrate ratios. There is considerable precedent in the literature for coupling coefficients greater than one (e.g., refs. 17, 19, 22, 31, 32, 37), including a value of 3 for alanine transport in the marine polychaete, *Glycera dibranchiata* [29].

Second, though it was apparent that Na^+ was required to support alanine and taurine uptake (Fig. 1), the absolute sensitivity of the transport sites to Na^+ was comparatively low; i.e., the K_{Na}'s for activation of transport by Na^+ were on the order of 325 mM (Fig. 7). Studies of mammalian Na-cotransport processes report "typical" K_{Na} values for Na^+ of between 30 and 80 mM (e.g., ref. 10). Furthermore, harmaline, a competitive inhibitor at the Na^+ binding site(s) of Na-cotransporters [4] displayed an apparent K_i of approximately 40 μM despite the presence of 425 mM of "competing" Na in the test solution (Fig. 6). In contrast, apparent K_i's for harmaline in mammalian systems are usually on the order of 1 mM [4], though Na^+ concentrations in such studies are only about 100 mM. Taken together, these observations suggest that the Na^+ binding sites of the gill's integumental transporters have an unusually low affinity for Na^+. The high concentration of Na^+ in seawater ($\simeq$425 mM) should cause essentially complete activation of amino acid influx. However, reduction of ambient Na^+, as would occur during transient exposures to reduced salinity, could play an important role in modulating this uptake.

Finally, the Na-activation of uptake in the gill was due primarily to an increase in the apparent affinity of gill transporters for substrate (Fig. 4); a similar pattern has been reported to occur in several Na-dependent transport systems, including alanine uptake into the mammalian intestinal mucosa [10].

It is instructive to consider the ability of gill cells to maintain 0.1 M cytoplasmic concentrations of amino acid in the face of constant exposure to a medium containing <1 μM amino acid in terms of the general "pump-leak" hypothesis [28]. The evidence presented here supports the idea that the amino-acid "pumps" of the apical membrane have kinetic characteristics that favor a relatively rapid influx of substrate against extreme chemical gradients. However, maintenance of the large cytoplasmic levels of amino acid implies that the "leak," carrier-mediated and passive, be very small. A low carrier-mediated efflux of amino acid may be the result of the requirement of large Na^+ concentrations to activate transporters; cytoplasmic Na^+ levels are likely to be ten times below that of the apparent K_{Na}'s of the integumental carriers. Recent evidence also suggests that the passive permeability of the apical membrane to amino acids is extremely low; permeability coefficients for taurine, for example, are on the order of 10^{-10} cm/sec [39]. Working in concert with one another, the "passive" and "active" transport characteristics of the gill epithelium appear to result in an extreme case of the pump/leak analogy, wherein the pump is allowed to approach its thermodynamic limit of uphill solute movement.

In conclusion, the maintenance of the characteristically large intracellular free amino-acid pool of the epithelial cells of *Mytilus* gill involves carrier-mediated, Na-dependent transport processes in the apical membrane of the gill. Though the chemical gradients sustained by these processes are extreme, the energy in the Na^+ electrochemical gradient is sufficient to drive transport if as few as 3 to 4 Na^+ ions are coupled to the transport of each amino acid. Maintenance of these solute gradients can be explained by the kinetic characteristics of the transporter, in conjunction with the asymetric distribution of Na^+ across the apical membrane and the low passive permeability of this membrane to amino acids. The nutritional potential of these integumental transport pathways seems clear [35]. It remains to be determined if this transport also plays a role in the modulation of cellular amino acid concentrations associated with cell volume regulation.

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