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Volume-Sensitive Taurine Transport in Fish Erythrocytes

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Summary. Taurine plays an important role in cell volume regulation in both vertebrates and invertebrates. Erythrocytes from two euryhaline fish species, the eel (*Anguilla japonica*) and the starry flounder (*Platichthys stellatus*) were found to contain high intracellular concentrations of this amino acid (≈30 mmol per liter of cell water). Kinetic studies established that the cells possessed a saturable high-affinity Na^+-dependent β-amino-acid transport system which also required Cl^- for activity (apparent K_m (taurine) 75 and 80 μM; V_{max} 0.85 and 0.29 μmol/g Hb per hr for eel (20°C) and flounder cells (10°C), respectively. This β-system operated with an apparent Na^+/Cl^-/taurine coupling ratio of 2 : 1 : 1. A reduction in extracellular osmolarity, leading to an increase in cell volume, reversibly decreased the activity of the transporter. In contrast, low medium osmolarity stimulated the activity of a Na^+-independent nonsaturable transport route selective for taurine, γ-amino-*n*-butyric acid and small neutral amino acids, producing a net efflux of taurine from the cells. Neither component of taurine transport was detected in human erythrocytes. It is suggested that these functionally distinct transport routes participate in the osmotic regulation of intracellular taurine levels and hence contribute to the homeostatic regulation of cell volume. Volume-induced increases in Na^+-independent taurine transport activity were suppressed by noradrenaline and 8-bromoadenosine-3′, 5′-cyclic monophosphate, but unaffected by the anticalmodulin drug, pimozide.

Key Words volume-sensitive fluxes · amino-acid transport · taurine · erythrocyte · adenosine-3′, 5′-cyclic monophosphate · fish

Introduction

Amino-acid transport in mammalian and avian erythrocytes has been extensively studied over the last 25 years, resulting in the identification of a number of functionally distinct amino-acid transporters each with its own characteristic substrate specificity, cation/anion dependence, kinetic properties, species distribution and inhibitor susceptibility (*see*, for example, refs. 9, 56, 58). Physiologically, erythrocyte amino-acid transport has been shown to be important for the provision of amino-acid precursors for intracellular glutathione biosynthesis [15, 17, 20, 33, 39, 40, 48, 57, 60, 61]. Erythrocyte amino-acid transporters also function as efflux routes for amino acids produced by protein degradation during reticulocyte maturation and perhaps also by hydrolysis of small peptides entering the cell from the plasma [18, 34–36, 47]. Amino-acid transport across the erythrocyte plasma membrane is required for the development of the intracellular stages of mammalian and avian malarial parasites [27, 44], and there is some evidence that erythrocytes may participate in interorgan transport of amino acids [8].

In contrast, there is surprisingly little information on the biochemistry or physiology of amino-acid transport in fish red cells. Unlike mammalian or avian erythrocytes, these cells are characterized by the presence of high intracellular levels of taurine [5, 26, 28], an amino acid which plays a widespread role in cell volume regulation in vertebrates [21] as well as invertebrates [42]. This ability of cells to utilize amino acids to regulate their volume in response to changes in extracellular osmolarity is an important homeostatic mechanism, especially in euryhaline species. Thus, Fugelli and co-workers have shown that erythrocytes (and other tissues) from the European flounder (*Platichthys flesus*) contain high intracellular levels of taurine (and GABA) which fall both *in vivo* and *in vitro* in response to hypoosmotic stress [22–26, 51, 52]. Quantitatively, this decrease in amino acids accounts for about 45% of the total intracellular osmolarity reduction occurring during the volume regulatory response, the remainder being contributed by selective volume-sensitive KCl efflux from the cell [24–26].

The amino acids lost from flounder red cells under hypotonic conditions can be quantitatively recovered from the extracellular medium [22, 23]. This observation has focused attention on the possible role of the plasma membrane in the regulation of

intracellular taurine (and GABA) levels, and it has been demonstrated that exposure of flounder red cells to hypoosmotic stress leads to an increase in their permeability to taurine [24, 25]. Similar changes in taurine transport in response to hypoosmotic stress have been observed in Ehrlich ascites tumor cells, a mammalian cell line which also utilizes taurine in cell volume regulation [30]. In the present study we have undertaken a detailed kinetic investigation of volume-sensitive taurine transport in erythrocytes from two euryhaline fish species, the eel (*Anguilla japonica*) and the starry flounder (*Platichthys stellatus*). We provide evidence that taurine levels in these cells are modulated by the balance between two functionally distinct volume-sensitive amino-acid transport routes, a Na^+-dependent β-amino-acid transporter which also requires Cl^- for activity and a novel Na^+-independent efflux route which is selective for small neutral amino acids. Data are presented to suggest that the Na^+-independent system is suppressed by cAMP.

Materials and Methods

Reagents and Isotopes

[1-^{14}C] Glycine, uniformly labeled L- and D-[^{14}C] amino acids, GABA and taurine were purchased from Amersham International PLC, Amersham, Bucks., U.K. Pimozide (a gift from Dr. C.M. Lee, The Chinese University of Hong Kong) was added to cell suspensions from a stock solution in dimethyl sulfoxide. The sodium and potassium salts of methylsulfuric acid were obtained from Hopkins and Williams, Chadwell Heath, Essex, U.K. The organic reagent *n*-dibutylphthalate was purchased from E. Merck, Darmstadt, F.R.G. 8-Br-cAMP (free acid) was obtained from Sigma Chemical Co., St. Louis, Mo., and was added to incubations as the monopotassium salt.

Fish

Eels (*Anguilla japonica*) were obtained locally in Hong Kong and maintained in fresh water aquaria. Starry flounders (*Platichthys stellatus*) were provided by the Bamfield Marine Station, British Columbia and held in running seawater. Fish were not fed while in captivity.

Cell Collection and Preparation

Fish were bled from the caudal artery into heparin. Blood from several fish was pooled. Erythrocytes were washed three times with a medium containing 150 mM NaCl, 5 mM glucose and 15 mM MOPS (titrated to pH 7.5 at 10 or 20°C with KOH). The buffy coat was discarded. The washed cells were resuspended to a hematocrit of approximately 10% and left overnight at 4°C. This was to ensure that the cells were at a steady state with respect to the incubation medium and not in a catecholamine-stimulated condition [3]. The hemoglobin content of cell suspensions was measured as described below.

Cell Volume and Hemoglobin Measurements

Cell volume was determined directly in capillary tubes using a microhematocrit centrifuge [13]. The volume of the cells in hypotonic medium was expressed as a relative cell volume (RCV) to that determined in isotonic NaCl or KCl medium as appropriate (RCV = 1.00). The hemoglobin contents of cell suspensions were measured as cyanmethemoglobin at 540 nm. Under isotonic NaCl conditions, the hemoglobin contents of packed fish and human erythrocytes were 200 and 250 g hemoglobin/liter of cells, respectively.

Transport Measurements

Influx Under Isotonic Conditions

These experiments were performed using a standard isotonic incubation medium of 150 mM NaCl or KCl, 5 mM glucose and 15 mM K^+-MOPS (pH 7.5) at 20°C for eel erythrocytes and 10°C for flounder red cells.

To measure the time course of [^{14}C]-taurine uptake by fish erythrocytes, equal volumes of washed cell suspension and incubation medium containing the appropriate concentration of radioactive permeant were preequilibrated to the incubation temperature and then mixed together [59]. Incubations were stopped at predetermined time intervals (1 min to 8 hr) by transferring 0.2 ml of the cell suspension (10% hematocrit) to a microcentrifuge tube (volume 1.5 ml) containing 0.8 ml ice-cold incubation medium layered on top of 0.5 ml ice-cold *n*-dibutylphthalate. The tube was immediately centrifuged in an Eppendorf 5414 microcentrifuge at 15,000 × *g* for 10 sec, and the aqueous and *n*-dibutylphthalate layers removed by suction, leaving the cell pellet at the bottom of the tube. After carefully wiping the inside of the centrifuge tube with absorbent tissue paper, the cell pellet was lysed with 0.5 ml 0.5% (vol/vol) Triton X-100 in water and 0.5 ml 5% (wt/vol) trichloroacetic acid was added. The precipitate was removed by centrifugation (15,000 × *g* for 2 min) and an aliquot of the protein-free supernatant counted for radioactivity by scintillation spectroscopy with appropriate quench correction. Blanks were obtained by processing cell samples which had been mixed with radioactive permeant at 0°C and immediately centrifuged. Transport values were calculated after subtraction of these blanks.

An alternative method of separating cells from extracellular medium was used in experiments where the initial rate of amino-acid uptake was measured [59]. Here, equal volumes of precooled (ice-cold) cell suspension and radioactive amino-acid solution were mixed in microcentrifuge tubes on ice and the incubations initiated by transferring the tubes into a water bath at 10 or 20°C. Incubations (typically 15 min) were stopped by transferring the tubes back into the ice-bath for a further 15 min. The cells were rapidly washed four times by centrifugation (15,000 × *g* for 10 sec) with 1-ml portions of ice-cold incubation medium and the washed cell pellets processed as described above.

Influx Under Hypotonic Conditions

In order to compare amino-acid transport in isotonic and hypotonic incubation media, a modification of the above methodology was used. Here, aliquots of cell suspension in isotonic medium were centrifuged (15,000 × *g* for 10 sec) and the supernatant liquid was removed. The cell pellets were kept on ice and an aliquot of ice-cold iso- or hypotonic incubation medium (containing the appropriate concentration of radiolabeled permeant) was added to give a final hematocrit of approximately 10%. The tubes were mixed and transferred to a water bath. Subsequent incubation, washing and cell processing procedures were as described above.

Efflux

Washed eel erythrocytes were incubated at a hematocrit of 30% for 1 hr (20°C) in 150 mM NaCl medium containing 0.4 μCi/ml [^{14}C]-taurine (final concentration 4 μM). The cells were rapidly washed free of extracellular radioactivity and Na^+ (six washes with ice-cold 150 mM KCl medium) and resuspended in ice-cold Na^+-free medium to a hematocrit of 20%. [^{14}C]-Taurine efflux at 20°C was initiated by mixing an aliquot of the ice-cold cell suspension with prewarmed incubation medium at different KCl concentrations representing 25 and 50% dilutions of the isotonic medium. At predetermined time intervals, 1-ml portions of cell suspension (final hematocrit 2%) were removed into ice-cold microcentrifuge tubes containing 0.2 ml *n*-dibutylphthalate and the cells rapidly sedimented below the oil (15,000 × *g* for 20 sec). A sample of supernatant liquid was removed for scintillation counting. The initial intracellular tracer level was measured by processing an aliquot of the 20% 'loaded' cell suspension as described for *influx*.

Amino-Acid Analysis

Cell pellets and plasma were deproteinized with 5% (wt/vol) sulfosalicyclic acid [46] and analyzed on an LKB 4400 automated amino-acid analyzer using the manufacturer's recommended lithium buffer system for physiological fluids.

Abbreviations

8-Br-cAMP: 8-bromoadenosine-3′, 5′-cyclic monophosphate; EGTA: ethylene glycol bis-(β-aminoethylether)-N_iN_i-tetraacetic acid; GABA: γ-amino-*n*-butyric acid; $MeSO_4^-$: methyl sulfate; MOPS: morpholine propane sulfonic acid; RCV: relative cell volume.

Results

Amino-Acid Levels in Fish Erythrocytes

Amino-acid analyses of eel and flounder erythrocytes established that the free amino-acid pool of these cells consisted mainly of taurine (25 to 45 mmol/liter cell water). These values contrast markedly with measured plasma taurine levels for both species in the region of 0.1 to 0.3 mM. Of the other amino acids detected, only GABA was found in high concentration (approximately 17 mmol/liter cell water) in flounder red blood cells. In contrast, no GABA was detected in eel erythrocytes. The high intracellular concentrations of taurine found in eel and starry flounder erythrocytes are comparable with those found in red blood cells of the European flounder [26] and Ehrlich ascites tumor cells [29]. Subsequent experiments in the present study focused on a kinetic analysis of taurine transport in eel and starry flounder erythrocytes, both under isotonic and hypotonic conditions.

Transport Under Isotonic Conditions

Time Course of Taurine Uptake

[^{14}C]-Taurine uptake by eel erythrocytes was measured at an approximate plasma concentration of 0.2 mM and 20°C, in both 150 mM NaCl and 150 mM KCl media. Taurine transport was almost completely Na^+ dependent (98%) and linear with time during the experimental period of 60 min, reaching a value in NaCl medium of 0.52 μmol/g Hb. Extension of the incubation period to 8 hr resulted in intracellular [^{14}C]-taurine levels reaching approximately 5 μmol/g Hb (1.67 mmol/liter cell water) demonstrating that eel erythrocytes are capable of concentrating tracer. Taurine uptake (0.2 mM extracellular concentration) by flounder red cells was also linear with time (0.15 μmol/g Hb after 3-hr incubation, 10°C) and Na^+-dependent (83%). Subsequent experiments measuring initial rates of taurine transport used incubation periods of 15 to 60 min.

Concentration Dependence of Taurine Uptake

To investigate the kinetics of Na^+-dependent taurine transport, the concentration-dependence (0.05 to 1 mM) of taurine uptake was measured in both eel (20°C) and flounder (10°C) erythrocytes (Figs. 1*A* and *B*, respectively). Uptake was resolved into Na^+-dependent and Na^+-independent components. Sodium-dependent uptake was saturable in both species' red cells. Kinetic constants estimated from *s/v versus s* plots of uptake in NaCl corrected for uptake in KCl medium were: K_m 75 and 80 μM; V_{max} 0.85 and 0.29 μmol/g Hb per hr for eel and flounder erythrocytes, respectively. Sodium-independent taurine uptake was nonsaturable, at a rate

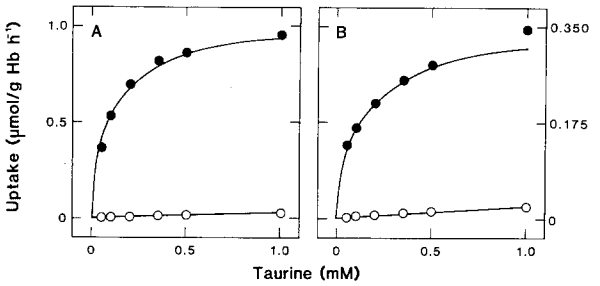

Fig. 1. Concentration-dependence of taurine uptake by fish erythrocytes. Initial rates of taurine uptake were measured in the presence (●) and absence (○) of Na^+ under isotonic conditions: (*A*) eel erythrocytes, 20°C; (*B*) flounder erythrocytes, 10°C. Values are the means of triplicate estimates. Kinetic constants for Na^+-dependent and Na^+-independent taurine transport are given in the text

of 0.025 μmol/g Hb per hr in both eel and flounder red blood cells at 1 mM extracellular taurine.

Cross-Inhibition Studies

To investigate the substrate specificity of the Na^+-dependent saturable component of taurine transport in eel erythrocytes, various amino acids were tested as inhibitors of taurine uptake in NaCl and KCl media (0.05 mM permeant, 1 mM inhibitor and 20°C). The results are presented in Table 1. Of the amino acids tested, only β-alanine was a highly effective inhibitor of Na^+-dependent taurine influx. The uptake of taurine by eel erythrocytes in the presence of varying concentrations of β-alanine is shown in Fig. 2. It can be seen that β-alanine inhibition of Na^+-dependent taurine influx was hyperbolic with respect to β-alanine concentration, half-maximal inhibition occurring at 0.6 mM β-alanine. Figure 2 also shows the effect of increasing concentrations of GABA on Na^+-dependent taurine transport by eel erythrocytes. GABA was a considerably less effective inhibitor of Na^+-dependent taurine uptake than β-alanine, half-maximal inhibition occurring at around 10 mM extracellular GABA. Complementary experiments with flounder erythrocytes gave results similar to those shown in Fig. 2. Thus, β-alanine was a more effective inhibitor of Na^+-dependent taurine uptake than GABA (50% inhibition occurring at 0.1 and 8 mM, respectively, taurine concentration = 0.025 mM, 10°C).

Parallel studies with eel erythrocytes established the presence of Na^+-dependent GABA transport (apparent K_m 9 mM) in these cells. Sodium-dependent GABA influx was inhibited 50% by 0.15 mM extracellular taurine at a GABA concentration of 2.5 mM.

Table 1. Substrate specificity of Na^+-dependent taurine transport in eel erythrocytes[a]

	Taurine uptake (nmol/g Hb per hr)			
	NaCl	KCl	Δ	Inhibition (%)
Control	432 ± 3	10.0 ± 0.15	422 ± 3	—
Glycine	403 ± 6	12.5 ± 1.25	390 ± 6	7.5
Sarcosine	409 ± 5	14.0 ± 0.75	395 ± 5	6.4
L-Proline	437 ± 4	12.0 ± 0.30	425 ± 4	0
β-Alanine	143 ± 6	10.0 ± 0.25	133 ± 6	68.5
GABA	387 ± 27	10.0 ± 0.95	377 ± 27	10.7
L-α-amino-*n*-butyric acid	436 ± 1	12.0 ± 0.40	425 ± 1	0
L-2,4-diamino-butyric acid	395 ± 5	13.0 ± 0.10	382 ± 5	9.5
L-Alanine	439 ± 6	10.5 ± 0.15	427 ± 6	0
L-Leucine	428 ± 12	11.5 ± 0.65	417 ± 12	1.4
L-Glutamate	433 ± 19	11.5 ± 0.50	422 ± 19	0
L-Lysine	432 ± 10	11.0 ± 0.80	421 ± 10	0

[a] Taurine and inhibitors were present at concentrations of 0.05 and 1 mM, respectively. Δ Is the difference between NaCl and KCl uptake rates. Values are the means ± SEM of triplicate estimates.

Kinetics of Na^+ Dependence

To explore Na^+-dependent taurine transport by fish erythrocytes in more detail, the initial rate of taurine uptake (0.05 mM, 20°C) by eel erythrocytes was measured as a function of extracellular Na^+ concentration (Fig. 3*A*). A sigmoid relationship was observed between taurine influx and Na^+ concentration. Hill-type analysis of the data (Fig. 3*B*) revealed a Hill coefficient of 1.94 with a K_{50} value of 85 mM Na^+. In flounder red cells, a similar sigmoid relationship between taurine transport and extracellular Na^+ was observed, with a measured Hill coefficient of 2.08.

Cl^- Dependence of Taurine Uptake

Sodium-dependent taurine transport by both eel and flounder erythrocytes was also found to be Cl^- dependent. As shown in Table 2, replacement of Cl^- in the incubation medium with $MeSO_4^-$ abolished Na^+-dependent taurine transport activity in eel erythrocytes. In contrast, the Na^+-independent component of taurine transport did not require Cl^- for activity, either under isotonic or hypotonic conditions. The relationship between Cl^- concentration and taurine uptake (0.05 mM) by eel red cells was hyperbolic (Fig. 3*A*), giving a K_{50} value of 57 mM Cl^- and a Hill coefficient of 1.07 (Fig. 3*B*).

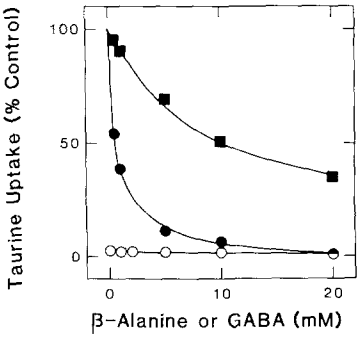

Fig. 2. Effects of β-alanine and GABA on taurine uptake by eel erythrocytes. [^{14}C]-Taurine uptake (0.05 mM, 20°C) was measured in the presence of nonradioactive β-alanine (●) and GABA (■) in isotonic NaCl medium. (○) Represents taurine uptake in isotonic KCl medium (± inhibitors). Results are plotted as a percentage of control NaCl uptake values in the absence of inhibitor (474 nmol/g Hb per hr). Values are the means of triplicate estimates

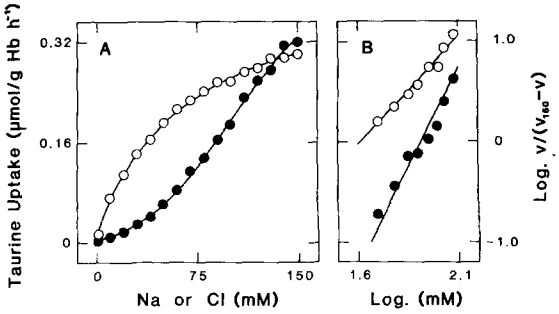

Fig. 3. Sodium and chloride dependence of taurine uptake by eel erythrocytes. The initial rate of [^{14}C]-taurine influx (0.05 mM, 20°C) was measured as a function of the concentration of Na^+ (●) or Cl^- (○) in the incubation medium (*A*). Isotonicity of the medium was maintained by balancing with KCl or $NaMeSO_4$, respectively. The order of Na^+/taurine and Cl^-/taurine interaction and the Na^+ and Cl^- concentrations reducing the flux by 50% (K_{50}) were estimated using a Hill-type analysis (*B*)

Table 2. Cation/anion-dependence of taurine transport in eel erythrocytes[a]

Medium	Uptake (nmol/g Hb per hr)
NaCl	444 ± 9.0
$NaMeSO_4$	37 ± 1.3
KCl	18 ± 0.5
$KMeSO_4$	19 ± 0.1

[a] Initial rates of [^{14}C]-taurine uptake were measured at 0.2 mM and 20°C. Values are the means ± SEM of triplicate estimates.

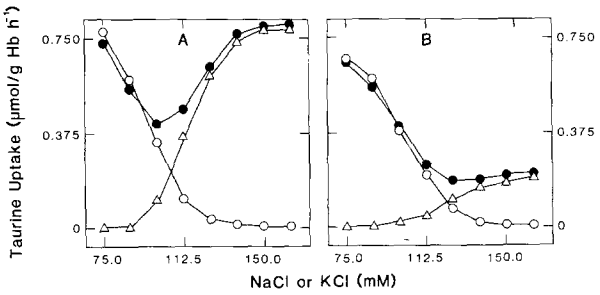

Fig. 4. Effects of osmolarity on the permeability of fish erythrocytes to taurine. Initial rates of [^{14}C]-taurine uptake (0.2 mM) were measured in solutions of different osmolarity, produced by diluting isosmotic NaCl (●) or KCl medium (○). (△) Is the difference between uptake in the presence and absence of Na^+: (*A*) eel erythrocytes, 20°C; (*B*) flounder erythrocytes 10°C. Values are the means of triplicate estimates

Transport Under Hypotonic Conditions

Effect of Medium Osmolarity on Taurine Transport

Eel and flounder erythrocytes exposed to hypoosmotic media (Fig. 4*A* and *B*, respectively) exhibited large changes in their permeability to taurine (0.2 mM). For example, in eel red blood cells, a 25% dilution of the extracellular isotonic medium (150 mM) led to a 13-fold increase in Na^+-independent taurine uptake and a 2.2-fold decrease in Na^+-dependent taurine transport activity. Under the same conditions, Na^+-independent taurine uptake by flounder erythrocytes increased by 26-fold, whereas Na^+-dependent transport activity was decreased fourfold.

Control experiments with eel erythrocytes (Table 3) established that the change in Na^+-independent taurine influx was fully reversible and triggered by the altered osmolarity of the medium, rather than by the decrease in ionic concentration. The loss of Na^+-dependent taurine transport activity observed under hypoosmotic conditions was also reversible (*see also* Table 3), and only partly due to the decrease in extracellular Na^+ and Cl^- concentrations.

In Fig. 5 we present results of experiments similar to those shown in Fig. 4, this time plotted as a function of cell volume. Erythrocyte volumes in the different NaCl and KCl media were measured relative to that in standard isotonic NaCl medium. The results reveal similar patterns to those shown in Fig. 4, and establish that a 5% increase in cell volume was sufficient to trigger significant changes in Na^+-dependent and Na^+-independent taurine transport activity.

As a control, we also studied the effect of osmolarity on taurine transport in human erythrocytes

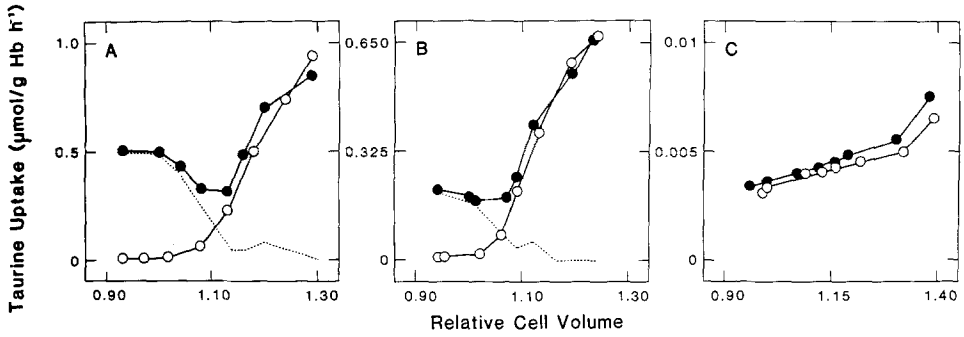

Fig. 5. Effects of cell volume on the permeability of eel, flounder and human erythrocytes to taurine. Initial rates of [^{14}C]-taurine uptake (0.2 mM, 20°C) by eel (*A*), flounder (*B*) and human (*C*) erythrocytes were measured in solutions of different osmolarity, produced by diluting isotonic media with distilled water. Results are plotted as a function of cell volume relative to that in 150 mM NaCl medium: (●), uptake in NaCl media; (○), uptake in KCl media. The broken lines (*A* and *B*) approximate the difference in uptake in NaCl and KCl media at the same relative cell volume. The eel and human erythrocyte experiments were performed at 20°C, and the flounder experiment at 10°C. Values are the means of triplicate estimates

Table 3. Effect of medium ionic concentration and osmolarity on taurine uptake by eel erythrocytes[a]

Medium	NaCl	KCl	Δ
150 mM	378 ± 11	8 ± 0.5	370 ± 11
112.5 mM	275 ± 4	100 ± 3	175 ± 5
75 mM	638 ± 3	615 ± 4	23 ± 5
112.5 → 150 mM	394 ± 5	8 ± 0.5	386 ± 5
75 → 150 mM	408 ± 4	12 ± 0.5	396 ± 4
112.5 + 75 mM sucrose	258 ± 3	8 ± 0.5	250 ± 3
75 + 150 mM sucrose	110 ± 4	7 ± 0.5	103 ± 4
112.5 NaCl + 37.5 mM KCl	265 ± 7		
75 NaCl + 75 mM KCl	161 ± 2		
112.5 NaCl + 37.5 mM $KMeSO_4$	227 ± 7		
75 NaCl + 75 mM $KMeSO_4$	120 ± 1		

[a] Initial rates of taurine uptake (nmol/g Hb per hr) were measured in media of different composition at 0.2 mM extracellular amino acid and 20°C. Δ is the difference in uptake in NaCl and KCl media, i.e., the Na^+-dependent component of transport. 112.5 → 150 mM and 75 → 150 mM refers to cells incubated for 1 hr in media containing 112.5 and 75 mM NaCl or KCl, respectively, followed by rewashing into standard isotonic medium in which uptake was measured. Values are the means ± SEM of triplicate estimates.

(0.2 mM extracellular concentration, 20°C). Taurine uptake in isotonic medium was slow, with no evidence of Na^+-dependence (3.35 ± 0.15 and 3.65 ± 0.15 nmol/g Hb per hr in 150 mM NaCl and KCl media, respectively). Swelling of human erythrocytes in hypotonic NaCl and KCl media resulted in changes in cell volume comparable to fish red cells (Fig. 5*C*), but with minimal increases in their permeability to taurine compared to those observed with eel and flounder cells.

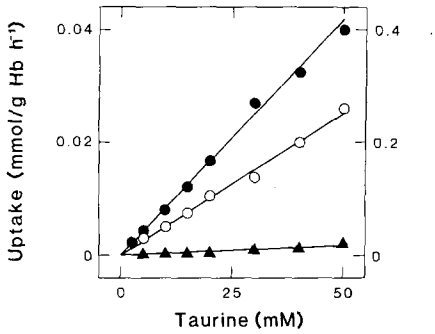

Fig. 6. Concentration-dependence of Na^+-independent taurine uptake by eel erythrocytes. Initial rates of taurine influx were measured at 20°C in media of different osmolarity in the absence of Na^+. Left-hand axis; uptake in 112.5 mM KCl (○) and 150 mM KCl media (▲). Right-hand axis; uptake in 75 mM KCl medium (●). Values are the means of triplicate estimates

Concentration Dependence of Na^+-Independent Taurine Transport

To investigate the Na^+-independent component of taurine transport in fish erythrocytes in more detail, subsequent experiments were performed in the absence of Na^+. Thus, in Fig. 6 the initial rate of taurine uptake by eel erythrocytes was measured as a function of extracellular taurine concentration in 150, 112.5 and 75 mM KCl media at 20°C. The high taurine concentrations (2.5 to 50 mM) used in the experiment made a significant contribution to the osmolarity of the medium. Therefore, allowance was made for this by making appropriate adjustments to the KCl concentrations of the incubation media. Uptake of taurine was very slow and linear with respect to concentration in isotonic medium

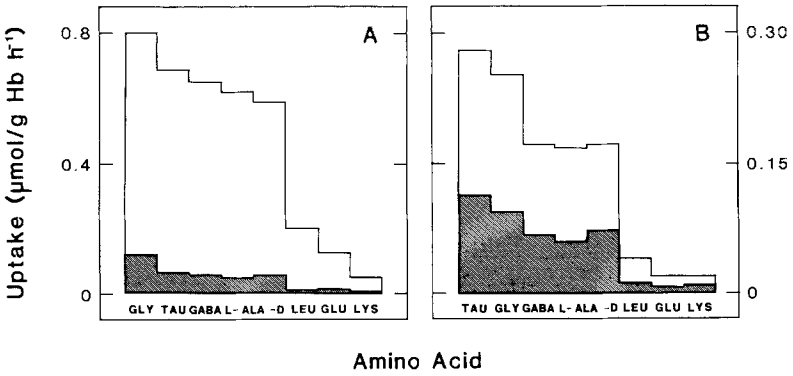

Fig. 7. Substrate specificity of Na^+-independent amino-acid uptake by fish erythrocytes. Initial rates of amino-acid uptake (0.2 mM) were determined in media containing 112.5 mM KCl and 75 mM KCl. Data are presented as the difference in uptake measured in 150 mM KCl medium and that measured upon dilution of the medium by 25% (hatched columns) and 50% (open columns): (*A*) eel erythrocytes, 20°C; (*B*) flounder erythrocytes, 10°C. Values are the means of triplicate estimates

(1.9 μmol/g Hb per hr at 50 mM extracellular taurine). In contrast, large increases in permeability to taurine were observed in 112.5 and 75 mM hypotonic media, this increased transport activity also exhibiting a linear concentration dependence (24 and 396 μmol/g Hb per hr at 50 mM taurine, respectively). Similar results were obtained with flounder erythrocytes at 10°C. Thus, taurine uptake was linear with concentration in 150 and 112.5 mM KCl media (1.6 and 42 μmol/g Hb per hr, respectively, at 50 mM extracellular taurine).

Substrate Specificity of Na^+-Independent Transport

To test whether these large Na^+-independent fluxes exhibited by fish erythrocytes in media of low osmolarity were selective for particular amino acids, the uptake of a series of [^{14}C]-labeled amino acids (0.2 mM extracellular concentration) by eel (Fig. 7*A*, 20°C) and flounder (Fig. 7*B*, 10°C) erythrocytes was measured in 150, 112.5 and 75 mM KCl media. Hypoosmotic conditions dramatically increased the permeability of both species erythrocytes to glycine, GABA and L- and D-alanine as well as taurine. In contrast, much smaller changes in permeability were observed for L-leucine, L-glutamate and L-lysine.

Na^+-Independent Taurine Efflux

Figure 8 shows the efflux of intracellular [^{14}C]-taurine from eel erythrocytes as a function of time. Efflux measured into isotonic medium at 20°C was slow over the experimental period of 40 min. Dilution of the isotonic medium by 25 and 50% resulted in eight- and 50-fold increases in the initial rate of taurine efflux from the cells.

Direct amino-acid analyses confirmed that there

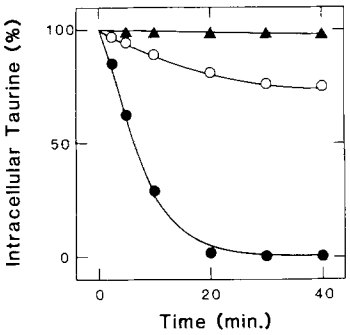

Fig. 8. Sodium-independent taurine efflux from eel erythrocytes. [^{14}C]-Taurine efflux was measured at 20°C into 150 (▲), 112.5 (○) and 75 mM (●) KCl media. The initial intracellular taurine concentration was 31.5 mmol/liter cell water. See Materials and Methods for other experimental details

was a net loss of taurine from the cells. This taurine was recovered in the extracellular medium. [^{14}C]-Taurine efflux observed in hypotonic medium was not further stimulated by the presence of 50 mM extracellular nonradioactive taurine, suggesting that the volume-sensitive Na^+-independent taurine transport route is unable to participate in accelerative exchange diffusion [45].

Effect of Pimozide on Na^+-Independent Taurine Transport

Calcium and calmodulin have been implicated in the regulation of volume-induced KCl and taurine release from Ehrlich ascites cells [31, 38]. The possible role of Ca^{2+} in the regulation of fish erythrocyte taurine transport was investigated by studying the effect of pimozide, an inhibitor of calmodulin function [53], on taurine transport under hypotonic conditions. At its solubility limit of 20 μM, pimozide had no detectable effect on volume-sensitive Na^+-

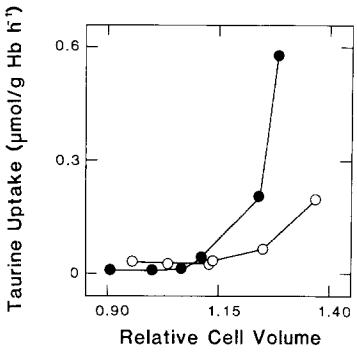

Fig. 9. Effect of 8-Br-cAMP on Na^+-independent taurine transport in flounder erythrocytes. Cells were incubated at 10°C (10% hematocrit) in isotonic NaCl medium in the absence (●) and in the presence of 10 mM 8-Br-cAMP (○). After 1 hr, extracellular Na^+ was removed by rapidly washing the cells with ice-cold isotonic KCl medium. Initial rates of [^{14}C]-taurine uptake (0.2 mM, 15 min flux) were measured at 10°C in the absence and presence of 8-Br-cAMP in solutions of different osmolarity (170, 150, 137.5, 125, 112.5 and 100 mM KCl media). Relative cell volume was determined after parallel incubations in the absence of [^{14}C]-taurine. Values are the means of duplicate estimates

independent taurine uptake by flounder and eel erythrocytes (*data not shown*).

Effect of 8-Br-cAMP on Na^+-Independent Taurine Transport

Cation transport in fish erythrocytes is subject to dual control by cell volume and catecholamines [3, 4]. We therefore tested the effect of 8-Br-cAMP, a phosphodiesterase-resistant cAMP analog, on hypotonic stimulation of Na^+-independent taurine transport. As shown in Fig. 9, treatment of flounder red cells with 10 mM cyclic nucleotide for 1 hr at 10°C produced a small increase in basal Na^+-independent permeability to taurine, but a marked inhibition of the hypotonic response. Thus, at an RCV of 1.28, taurine uptake by control cells was 576 nmol/g Hb per hr compared with 10 nmol/g Hb per hr at RCV 1.00, a difference of 566 nmol/g Hb per hr. The corresponding increase in erythrocyte permeability to taurine for 8-Br-cAMP-treated cells can be estimated from Fig. 9 to be approximately 70 nmol/g Hb per hr, i.e., an inhibition of 88%. As expected, the 8-Br-cAMP-treated cells had consistently higher cell volumes than the untreated controls.

It was established that the effect of 8-Br-cAMP (10 mM) on taurine transport was proportional to the length of time cells were pre-incubated with cyclic nucleotide, presumably reflecting the slow diffusion rate of 8-Br-cAMP across the cell membrane

Table 4. Effects of noradrenaline on flounder erythrocyte volume and Na^+-independent taurine uptake[a]

	Medium	RCV	Taurine uptake
Control	150 mM KCl	1.00	15 ± 1
Noradrenaline	150 mM KCl	1.37	57 ± 1
Control	87.5 mM KCl	1.37	959 ± 11

[a] Cells were incubated at 10°C (10% hematocrit) in isotonic NaCl medium in the absence (control) and in the presence of 10 μM noradrenaline. After 3 hr, extracellular Na^+ was removed by rapidly washing the cells with ice-cold isotonic KCl medium. Initial rates of taurine uptake (nmol/g Hb per hr) were measured in KCl media at 0.2 mM extracellular amino acid and 10°C. Noradrenaline was present during the KCl wash and flux assay of hormone-treated erythrocytes. RCV was determined after parallel incubations in the absence of [^{14}C]-taurine. Taurine uptake rates are means ± SEM of triplicate estimates.

at 10°C. Using a 3-hr pre-incubation period, we found that 1 mM 8-Br-cAMP suppressed volume-sensitive, Na^+-independent taurine transport by 50% (RCV 1.30). As might be expected from these results, noradrenaline-induced cell swelling under isotonic conditions, which is likely to be mediated by cAMP, was not associated with an increase in cellular permeability to taurine (Table 4). Thus, noradrenaline treatment of flounder erythrocytes (10 μM for 3 hr at 10°C) caused a 37% increase in cell volume, but only a modest 3.6-fold rise in Na^+-independent taurine transport. Dilution of control cells in 87.5 mM medium to produce the same increase in cell volume induced a 64-fold activation of Na^+-independent taurine transport activity.

Discussion

Fugelli and co-workers have documented the involvement of taurine in volume regulatory responses by erythrocytes from the European flounder [22, 24–26]. In the present study we have demonstrated high intracellular taurine concentrations in erythrocytes from two other euryhaline fish species, the eel and starry flounder. We have used cells from these two species to characterize the membrane transport mechanism(s) involved in the regulation of cellular permeability to taurine in response to cell expansion.

Taurine uptake by eel and flounder erythrocytes under isotonic conditions was shown to be mediated by a saturable high-affinity transport mechanism which required both Na^+ and Cl^- for activity. In this respect the taurine transporter in fish erythrocytes resembles that for glycine transport in pigeon [49] and human red blood cells [16],

as well as in rabbit [10, 55] and guinea pig reticulocytes [19]. Glycine transport in these cells is mediated by a specific transport system, designated system *Gly*, which requires both Na^+ and Cl^- for activity. In the pigeon erythrocyte, system *Gly* operates with a presumed Na^+/Cl^-/amino-acid coupling ratio of 2 : 1 : 1 [32, 50]. Hill-type analyses of the relationship between taurine transport activity and Na^+ and Cl^- concentration in fish erythrocytes suggest that the system also functions with an apparent Na^+/Cl^-/taurine coupling ratio of 2 : 1 : 1. With respect to substrate specificity, Na^+/Cl^--dependent taurine transport activity in fish erythrocytes was shown to be inhibited by β-alanine and, to a lesser extent, GABA but not by representative neutral α-amino acids. The substrate selectivity of the transporter therefore identifies it as a β-system similar to that present in pigeon erythrocytes and a variety of mammalian cells [6, 7, 14, 54]. Taurine-sensitive β-alanine transport in pigeon erythrocytes also exhibits a Na^+/amino acid coupling ratio greater than first-order [14, 54], while kinetic and energetic considerations suggest a 2 : 1 coupling ratio for Na^+-dependent taurine transport in Ehrlich ascites cells [30, 37]. Replacement of Cl^- by NO_3^- has been reported to cause 75% inhibition of taurine transport in Ehrlich ascites cells [38]. By analogy with system *Gly* [16], this may reflect an ability of NO_3^- to function as a partially active Cl^- substitute.

The presence of a concentrative Na^+-dependent β-system in both eel and flounder erythrocytes presumably contributes to the high intracellular taurine levels detected in these cells. It is therefore interesting that this system is apparently down-regulated when cells are exposed to hypoosmotic stress (Fig. 4). Loss of Na^+-dependent transport activity can only in part be attributed to the reduced extracellular concentrations of Na^+ and Cl^- in the medium (Table 3). The effect of lowered osmolarity on transporter activity is fully reversible and is therefore unlikely to be the result of general disruption of ion gradients across the cell membrane. Similar down-regulation of Na^+-dependent taurine transport under hypoosmotic conditions has been postulated for the Ehrlich cell [30], and may also occur in erythrocytes from the European flounder (Fig. 3, ref. 24).

GABA is also a major component of the amino-acid pool in flounder, but not in eel erythrocytes. Although GABA is a substrate for the β-system, its affinity for both the eel and flounder erythrocyte transporters is low. The reason for GABA accumulation in flounder, but not eel erythrocytes is therefore not immediately obvious, particularly since no GABA was detectable in plasma samples from either species.

As shown in Fig. 4, down-regulation of Na^+-dependent taurine transport activity under hypotonic conditions was paralleled by a dramatic increase in Na^+-independent permeability to taurine. This phenomenon was observed in erythrocytes from both the eel and flounder, the increased Na^+-independent permeability to taurine returning to normal levels when the medium osmolarity was restored. Thus, the effect of reduced osmolarity on Na^+-independent permeability to taurine cannot be attributed to nonspecific membrane damage resulting from cell expansion. Similar volume-dependent increases in Na^+-independent permeability to taurine have been observed in erythrocytes from the European flounder and in Ehrlich ascites cells [24, 30]. The increase in Na^+-independent permeability to taurine seen in the present study was shown to be species specific, no significant changes in permeability to taurine occurring when human erythrocytes were exposed to the same hypotonic media under identical conditions. This latter observation is additional evidence against the possibility of nonspecific membrane damage or opening of prelytic pores contributing to the observed increase in cellular permeability to taurine. Instead, we interpret our results to suggest that eel and flounder erythrocytes possess a volume-sensitive Na^+-independent channel or transporter which is activated under hypotonic conditions.

Further evidence to support this view comes from the finding that the increased cell permeability under hypotonic conditions is selective for particular amino acids, the specificity of the route being directed towards small neutral molecules (glycine, taurine, GABA) with apparently no preference for either L- or D-isomers of α-amino acids (alanine) (Fig. 7). The system discriminates against size and possibly charge (leucine, glutamate and lysine). It is striking that the substrate selectivity of this route is the same in both eel and flounder erythrocytes. The two major amino acids contributing to cell volume regulation in Ehrlich ascites cells are taurine and glycine (Table 1, ref. 30). However, the amino-acid selectivity of the volume-sensitive route present in these cells would appear to differ from the fish erythrocyte system in that glutamate as well as aspartate are lost from the cells during hypoosmotic stress. Kinetically, the fish system exhibited a linear concentration dependence for taurine influx up to 50 mM extracellular taurine. At the latter concentration, the rate of taurine influx through the system was very high (400 μmol/g Hb per hr in 50% diluted medium). Although it is more readily demonstrated under influx conditions, the physiological role of this system is presumably to mediate net amino-acid efflux from the cell. The data presented in Fig.

8 suggest that the system is symmetrical with respect to taurine influx and efflux. Thus, a 50% dilution of the extracellular medium caused the total loss of accumulated [^{14}C]-taurine from eel erythrocytes within 20 min at 20°C.

The experiments summarized in Table 3 indicate that the permeability of eel erythrocytes to taurine changes in response to a decreased medium osmolarity, i.e., an increase in cell volume. This also appears to be the case for taurine and glycine transport in Ehrlich ascites tumor cells [30]. An important question is whether the membrane components responsible for amino-acid transport respond directly to membrane expansion or whether regulation of transport activity is mediated by a secondary messenger(s), the intracellular levels of which change in response to alterations in cell volume. Ca^{2+} release from intracellular stores has been implicated in the activation of volume-sensitive KCl efflux from Ehrlich ascites cells and pimozide, a calmodulin antagonist [53], has been reported to inhibit this volume response with an IC_{50} value of 4 μM [31]. Similarly, it has been suggested that Ca^{2+} may also activate the taurine leak pathway in these cells [38]. In the present study, we found that 20 μM pimozide had no effect on the Na^+-independent taurine pathway in fish red cells exposed to hypotonic medium. Cation transport in trout [3, 4] and avian erythrocytes [41] are subject to dual regulation by cell volume and catecholamines. The effect of catecholamines on cation transport in avian (and presumably fish) cells is mediated by cAMP-dependent membrane protein phosphorylation [43]. The 8-Br-cAMP experiment presented in Fig. 9 suggests that volume-sensitive Na^+-independent taurine transport in fish erythrocytes may also be modulated by cAMP. Support for this hypothesis comes from the finding that noradrenaline-induced cell swelling was not associated with the expected large increase in Na^+-independent taurine transport (Table 4), cAMP inhibition presumably overriding the volume response. Catecholamine-induced Na^+ and K^+ influx and hence cell swelling occur physiologically in fish under stress [3, 12] and may serve to increase hemoglobin-oxygen affinity through intracellular alkalinization mediated by Na^+/H^+ exchange [1, 2, 11].

In conclusion, we interpret our results to indicate that eel and flounder erythrocytes possess a novel Na^+-independent amino-acid transporter or channel responsive to changes in cell volume. This transport route is not inhibited by pimozide and exhibits a selectivity towards small neutral amino acids, including taurine. The activity of the system is stimulated by an increase in cell volume. We have also established that the cells possess a volume-sensitive Na^+/Cl^--dependent β-amino-acid transporter which is reversibly inhibited under hypotonic conditions. It is therefore envisaged that intracellular taurine levels in fish erythrocytes are regulated in response to changes in extracellular osmolarity by the reciprocal control of taurine uptake by the β-system and taurine efflux *via* the Na^+-independent transport route, these two permeability pathways contributing to the homeostatic regulation of cell volume. Our results further suggest that cAMP may function as a negative modulator of the Na^+-independent system, permitting catecholamine-induced cell swelling to occur in the absence of taurine efflux.

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