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Taurine transporter in primary cultured neonatal rat heart cells: a comparison between cardiac myocytes and nonmyocytes

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Abstract

In the present study, we examined the characteristics of the taurine transporter and the intracellular taurine content in cultured neonatal heart cells. Primary cultures of cardiac myocytes and cardiac fibroblasts (nonmyocytes) were prepared from 1-day-old Wistar rats. The parameters examined were: (a) intracellular taurine content by the HPLC method, (b) the expression levels of taurine transporter mRNA and protein using northern and western blot analysis, and (c) transporter activity determined by the uptake of ^{3}H-labeled taurine. The taurine content of myocytes was significantly higher (3-fold) than that of nonmyocytes. Taurine transporter mRNA was strongly expressed in both myocytes and nonmyocytes, whereas the magnitude [normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression] of the transporter mRNA expressed in myocytes was lower than that in nonmyocytes. The expression level of transporter protein in myocytes was also lower than that of nonmyocytes. Uptake of radiolabeled taurine into monolayer cultures of heart cells was stimulated markedly by the presence of Na^+ in the medium, whereas this uptake was almost abolished in the absence of Na^+. The Na^+/taurine stoichiometry was 2:1 for both myocytes and nonmyocytes. Kinetic analysis showed that a single saturable system was involved in taurine uptake into both cell types. In myocytes, the apparent K_m and V_{max} values for the transporter were 20.7 ± 0.5 μM and 1.07 ± 0.01 nmol/10^6 cells/30 min, respectively. Similarly, those of nonmyocytes were 20.3 ± 0.7 μM and 0.42 ± 0.01 nmol/10^6 cells/30 min. These findings indicated that both myocytes and nonmyocytes expressed an identical taurine transporter with a Michaelis–Menten constant of 20–21 μM and that a higher taurine content in myocytes may be associated with a higher V_{max}.

Keywords: Taurine transporter; Cell culture; Fibroblasts; Gene expression; Membrane transport; Myocytes

1. Introduction

Taurine (2-aminoethanesulfonic acid) is by far the most abundant free amino acid in the mammalian heart, comprising in excess of 50% of the total free amino acid pool [1,2]. Although the physiological function remains undefined, taurine exhibits an extensive cardiovascular pharmacology. High intracellular levels are generated and maintained by biosynthesis and/or transmembrane transport processes according to the specific tissue, developmental stage, or nutritional status [1,2]. In humans, the ability to synthesize taurine is low compared with that of other species, including rats [1,2]. Immature animals cannot biosynthesize enough taurine in their systems and must depend upon a supply of taurine from outside. Namely, a taurine transporter is essential to the maintenance of adequate tissue levels of taurine. Therefore, we examined the characterization of the taurine transporter using neonatal cultures. The major source of myocardial taurine is via an active transport mechanism, although taurine is synthesized by the heart [1,2].

The taurine transporter has been cloned from MDCK (Madin–Darby canine kidney) cells [3], rat brain [4], mouse

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Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HPLC, high-performance liquid chromatography; MDCK cell, Madin–Darby canine kidney; Ac-LDL, acetylated-low density lipoprotein; NeuN, vertebrate neuron-specific nuclear protein; ANP, atrial natriuretic peptide; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; BSA, bovine serum albumin; IgG, immunoglobulin G; HRPE, human retinal pigment epithelium.

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brain [5], human FRTL-5 thyroid cells [6], human placenta [7,8], and human retinal pigment epithelial cells [9,10]. A comparison of nucleotide sequences of the taurine transporter cDNAs with those of other cloned transporters indicated that the taurine transporter belonged to a gene family that encodes Na^+- and Cl^--coupled transporters [2,3].

Cardiac myocytes occupy 75% of the structural space of the myocardium, but they constitute only one third of the cell population. The remaining cells include cardiac fibroblasts, neurons, endothelial cells, and vascular smooth muscle cells [11]. Although numerous cell types are present in the heart, cardiomyocytes and cardiac fibroblasts are the predominant cell types in the neonatal rat heart [11]. The present study was undertaken to clarify the physiological aspects of the cardiac taurine transporter using cultured myocytes and nonmyocytes prepared from neonatal rats.

2. Material and methods

2.1. Preparation of cardiac myocyte and nonmyocyte cultures

Preparation of primary cardiac myocyte and nonmyocyte cultures from 1-day-old Wistar rats was as described by Sadoshima and Izumo [12]. For selective enrichment of cardiac myocytes, dissociated cells were preplated for 1 hr, during which the nonmyocytes attached readily to the bottom of the culture dish [13]. Non-adherent cells, mostly myocytes, were plated at a density of 2–5 $\times$ 10^6 cells/mL/dish. Bromodeoxyuridine (0.1 mM) was added during the first 2 days to prevent proliferation of the nonmyocytes. This procedure yielded cultures with 90–95% myocytes, as assessed by microscopic observation of beating cells. Cells were kept in serum-containing culture medium, Dulbecco's modified Eagle's medium/F-12 (Dainippon Pharmaceutical Co., Ltd.; 1:1, v/v) supplemented with newborn calf serum (5 or 10%; Dainippon), 3 mM pyruvic acid, 100 μM ascorbic acid, 5 μg/mL insulin, 5 μg/mL transferrin, and 5 ng/mL of selenium (Boehringer Mannheim; DMEM/F-12 medium) for 48 hr followed by serum-free medium. All experiments were performed 24 hr after transferring the cells to serum-free medium. In nonmyocyte preparations, contaminating cells, such as endothelial cells, neurons, and smooth muscle cells were estimated by labeling with acetylated-low density lipoprotein (Ac-LDL) and immunostaining with a monoclonal antibody against α-smooth muscle actin and vertebrate neuron-specific nuclear protein (NeuN), respectively [14,15]. The nonmyocyte fractions consisted primarily of fibroblasts, since microscopic observation did not reveal beating cells and chemical staining showed that these fractions generally contained less than 10% positive cells (Table 1). Moreover, cardiac fibroblasts did not express either atrial natriuretic peptide (ANP) or Na^+–Ca^{2+}-exchanger mRNA, both used for the positive identification of cardiac myocytes, but did express collagen type I and fibronectin markers for fibroblasts, on northern blot analysis. Cell density was assessed with a hemocytometer.

Table 1
Purity of the nonmyocyte cell population

Stain	Positive cells/observed cells (%)
Ac-LDL	51/833 (6)
α-Smooth muscle actin	12/1065 (1)
NeuN	3/300 (1)

In nonmyocyte preparations, contaminating cells, such as endothelial cells, neurons, and smooth muscle cells were estimated by labeling with acetylated-low density lipoprotein (Ac-LDL) and immunostaining with a monoclonal antibody against α-smooth muscle actin and vertebrate neuron-specific nuclear protein (NeuN), respectively. The samples (N = 300–1065 cells) were obtained from three different primary culture preparations.

2.2. Intracellular taurine content

The intracellular taurine content of cardiocytes was measured using an HPLC system (JASCO880-PU equipped with an HITACHI F1000 fluorescence detector and HITACHI D-2500 integrator) as described by Jones and Gilligan [16]. A TSKgel ODS-80TS HPLC column (150 mm $\times$ 4.6 mm i.d.) was used with a flow rate of 1.2 mL/min. The column eluate was monitored at 340 nm (excitation) and 455 nm (emission). The mobile phase consisted of 40% methanol and 1% tetrahydrofuran in 0.1 M acetate buffer (pH 7.2). The cultured cells were rinsed three times with 0.5 mL of cold PBS, and then scraped off the dish with a cell scraper. To the cell suspension (~30 μg protein/1 mL) was added 10% trichloroacetic acid (0.25 mL), and the mixture was centrifuged for 10 min $\times$ 15,000 *g* at 4°. The supernatant (1 mL) was neutralized with 0.1 mL of 1 M NaOH. A 60-μL volume of the solution was treated with 40 μL of fluorescamine reagent for 30 sec at room temperature, and 40 μL of the solution was injected into the column within 1 min.

2.3. Isolation and northern blot analysis of RNA

Total RNA was extracted from cardiac cells (myocytes and nonmyocytes) using the guanidinium thiocyanate–phenol–chloroform extraction method [17]. The RNA concentration was determined spectrophotometrically by absorbance at 260 nm. Ethidium bromide staining of 18S and 28S RNA confirmed that an equal amount of RNA was loaded in each lane. Northern blot analysis was performed according to the procedure described by Kim *et al.* [18]. The cDNA probes used were as follows: canine taurine transporter cDNA, a 7-kb fragment (from Dr. S. Uchida, Medical School, Tokyo Medical and Dental University); and rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a 1.3-kb fragment. The cDNA probes were labeled with [^{32}P]dCTP (specific activity, 3 mCi/mmol; New England Nuclear) by random primer extension using a Bca BEST

Labeling Kit (Takara). Autoradiography was performed on a Kodak XAR-5 film with an intensifying screen at −80°. Autoradiograms were quantified using an image analyzer (BAS1500, Fuji Film Corp.). Results were normalized to GAPDH gene expression.

2.4. Western blot analysis

Taurine transporter protein levels were determined by western blot analysis [19]. After treatment with trypsin to remove the cells from the dishes, the cells were suspended in HEPES buffer (10 mM) containing 2 mM EDTA, 0.1 mg/mL of phenylmethylsulfonyl fluoride, and 1 mM sodium orthovanadate. After sonication, the cell lysates were centrifuged at 10,000 *g* for 15 min at 4°, and the supernatants (24 μg protein/lane) were loaded on 10% gels and were subjected to SDS–PAGE. The gels were electroblotted to nitrocellulose membranes that were subsequently blocked with 5% BSA at room temperature. The membranes were incubated with the rat taurine transporter antibody (Alpha Diagnostic) 1:1000, and after a washing step, were incubated with a secondary antibody, goat anti-rabbit IgG. After incubation with the secondary antibody, the western blots were developed using an enhanced chemiluminescence reagent (Amersham Pharmacia Biotech). The blots were stripped and reprobed with an anti-actin antibody (Sigma-Aldrich, Inc.) (1:400), and were analyzed with the aid of a computer program (Alpha Imager).

2.5. Uptake measurements

The uptake of taurine in cardiocytes was measured at 37°. The culture medium was removed from the dishes, and the cells were washed twice with uptake medium before the initiation of uptake measurements. The composition of the uptake medium was: 10 mM HEPES/Tris (pH 7.4), 150 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, and 10 mM glucose. After washing, the uptake medium containing [1,2-^{3}H]taurine (0.1 μCi/μL, 1 mL) was added to the dishes. In preliminary experiments, taurine uptake into the cells was linear for at least 2 hr. After incubation for 30 min the medium was removed, and the cells were quickly washed three times with cold (4°) tritium-free medium, after which 0.5 mL of 0.1 M NaOH was added to each dish. After 0.5 hr, the contents from the dishes were transferred to scintillation vials, and radioactivity was assessed by liquid scintillation spectrometry. The concentration dependence of taurine uptake was determined, the uptake activity being measured with 10, 15, 20, 50, and 100 μM taurine. In the experiments determining the effects of Na^+, choline was substituted for Na^+ in the uptake medium.

2.6. Statistics

Statistical significance was determined by Student's *t*-test or ANOVA (Bonferroni's method was used to compare individual data when a significant *F* value was shown) and the χ^2-test, depending upon the design of the experiments. Each value was expressed as the mean ± SEM. Differences were considered statistically significant when the calculated *P* value was less than 0.05.

3. Results

3.1. Comparison of intracellular taurine content between myocytes and nonmyocytes

We measured the intracellular taurine content in myocytes and nonmyocytes cultured in serum-free medium for 24–48 hr. The total protein content in myocytes and nonmyocytes was 136 ± 15 and 86 ± 10 μg/10^6 cells, respectively. The intracellular taurine content of both cell types is expressed as picomoles per microgram of protein and nanomoles per 10^6 cells (Table 2). The taurine content of myocytes (21 ± 2 nmol/10^6 cells) was significantly higher (3-fold) than that of nonmyocytes (7 ± 1 nmol/10^6 cells). Similarly, myocyte taurine per μg protein was 181 ± 21 pmol, 2-fold higher than nonmyocyte taurine (84 ± 14 pmol/μg protein).

3.2. Identity and characteristics of the taurine transporter on myocytes and nonmyocytes in culture

The heart regulates cytosolic taurine levels by several selective transport mechanisms, of which the taurine/Na^+ cotransport system is the most important [3,7]. We examined the characteristics of the taurine transporter in cultured neonatal rat heart cells (myocytes and nonmyocytes). As shown in Fig. 1, taurine transporter mRNA was strongly expressed in both myocytes and nonmyocytes, whereas the magnitude (normalized to GAPDH gene expression) of the transporter mRNA expressed in myocytes was lower than that in nonmyocytes. The expression levels of transporter protein in myocytes were also lower than those of nonmyocytes (Fig. 1).

To determine suitable conditions for characterizing taurine uptake, we first tested the linearity of taurine uptake into heart cells. Uptake increased linearly for at least 120 min (data not shown). Therefore, the uptake at 30 min was used as the initial rate. Uptake of radiolabeled taurine into

Table 2
Intracellular taurine concentration in neonatal rat heart cells

	Protein content	Taurine concentration	
	μg/10^6 cells	pmol/μg protein	nmol/10^6 cells
Myocytes	136 ± 15*	181 ± 21*	21 ± 2*
Nonmyocytes	86 ± 10	84 ± 14	7 ± 1

Values are means ± SEM of 6–15 samples obtained from 3 different cultures.
* $P < 0.01$ versus nonmyocytes.

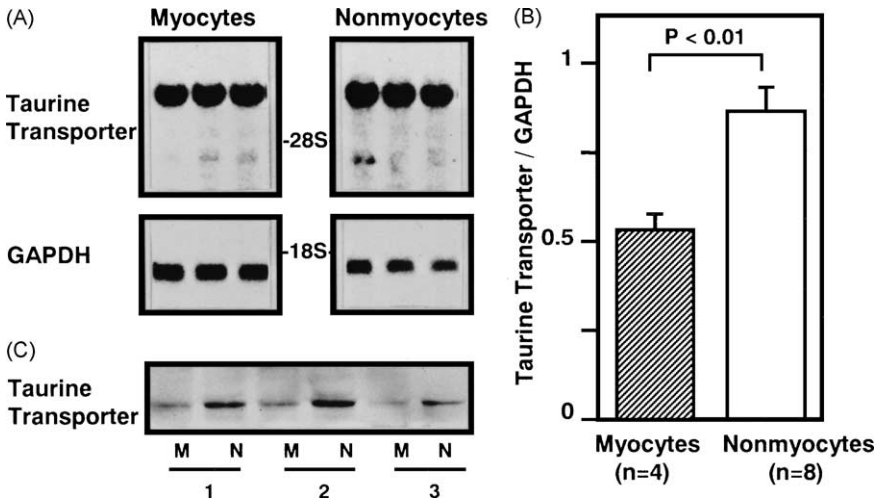

Fig. 1. Expression levels of taurine transporter mRNA and protein in neonatal rat heart cells. (A) Typical northern blot autoradiograms of myocyte and nonmyocyte mRNA for taurine transporter and GAPDH. The RNA concentration was determined spectrophotometrically by absorbance at 260 nm. Ethidium bromide staining of 18S and 28S RNA confirmed that an equal amount of RNA was loaded in each lane (data not shown). (B) Quantitation of mRNA/GAPDH ratio for taurine transporter mRNA levels in myocytes and nonmyocytes. Values are means ± SEM, obtained by densitometric analysis. (C) Western blot pattern of taurine transporter protein in myocytes (M) and nonmyocytes (N), obtained from three different experiments. Equal protein of 24 μg/lane was loaded onto a 10% SDS–polyacrylamide gel.

monolayer cultures of heart cells was stimulated markedly by the presence of Na^+ in the medium, whereas this uptake was almost abolished in the absence of Na^+ (Fig. 2). When the Na^+-dependent uptake of taurine was plotted against the Na^+ concentration, a sigmoidal curve was obtained (Fig. 3), suggesting that more than one Na^+ ion was associated with the transfer of one taurine molecule. To estimate the number of Na^+ ions involved in the process, the experimental data were fit to a Hill-type equation [10,19,20]. The Na^+:taurine coupling ratio determined by non-linear regression analysis was 1.8 ± 0.1 for myocytes and 1.9 ± 0.2 for nonmyocytes, respectively. The competition for taurine uptake by β-alanine is shown in Fig. 4. β-Alanine (0–250 μM) reduced the amount of taurine taken up into both myocytes and nonmyocytes. β-Alanine competitively inhibited taurine uptake into the heart cells. The K_i values of β-alanine in myocytes and nonmmyocytes were similar (about 30 μM).

The relationship between uptake rate and substrate concentration for taurine uptake was analyzed by the initial uptake rates (30-min incubation) at various concentrations of taurine (Fig. 5). In both cell types, the uptake rate was found to be saturable with increasing concentrations of taurine over a range of 0–100 μM and the plot of taurine concentration versus uptake rate was hyperbolic, apparently indicating participation of a single transport system in these uptake processes (Fig. 5). As shown in Fig. 5, the Eadie–Hofstee plot (uptake rate/taurine concentration versus uptake rate) was linear (myocytes: $r^2 = 0.95$; nonmyocytes: $r^2 = 0.69$), suggesting that a single system over a range of 0–100 μM was involved in taurine uptake in both myocytes and nonmyocytes. The kinetics parameters

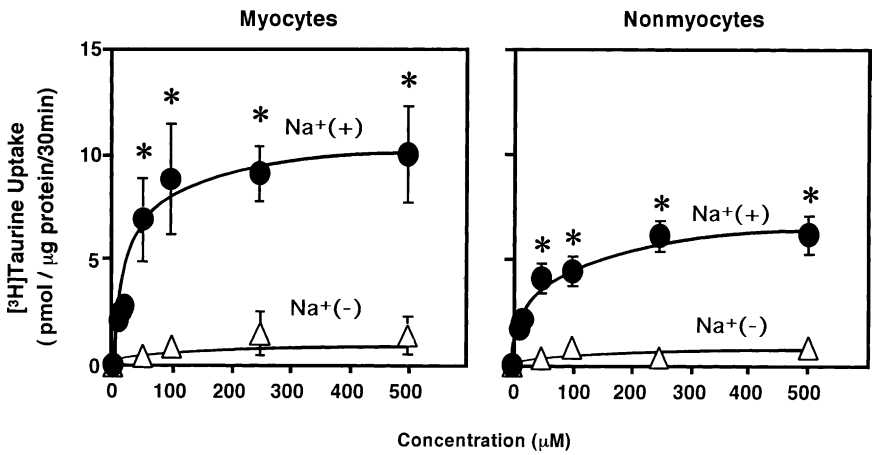

Fig. 2. Characteristics of taurine uptake in neonatal rat heart cells. Taurine uptake was measured in cells incubated for 30 min with and without NaCl. Values are means ± SEM of 4–12 samples obtained from 3 different cultures. Key: (*) $P < 0.01$ versus the buffer without NaCl at each concentration.

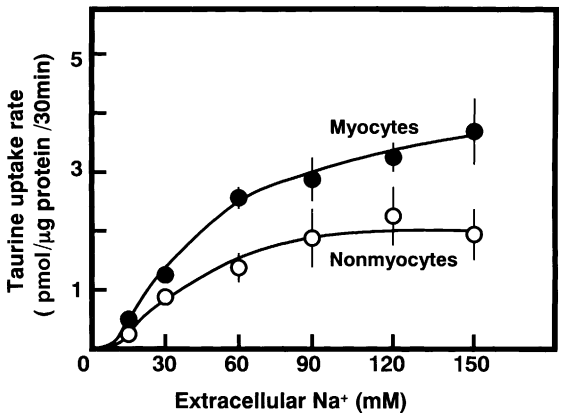

Fig. 3. Sodium-dependent taurine transport by neonatal rat heart cells. Taurine transport in neonatal rat heart cells was quantified as a function of extracellular sodium concentration. Each point represents the mean ± SEM; N = 6 for myocytes and for nonmyocytes. Non-linear regression analysis using the modified Hill equation was used to construct the given curves with coefficients of 1.8 and 1.9 for myocytes and nonmyocytes, respectively.

of taurine uptake, the K_m and V_{max} values, determined by linear regression were 20.7 ± 0.5 μM and 1.07 ± 0.01 nmol/10^6 cells/30 min for myocytes and 20.3 ± 0.7 μM and 0.42 ± 0.01 nmol/10^6 cells/30 min for nonmyocytes, respectively. We also obtained similar K_m and V_{max} values for the taurine transporter by a Lineweaver–Burk plot (data not shown).

4. Discussion

When considering taurine and the heart, the central questions concern the mechanism by which the relatively large gradient for taurine is established across the sarcolemmal membrane and the purposes to which it is put by the heart [21]. Carnivores are particularly susceptible to taurine deficiency because of their poor capacity to synthesize taurine and the obligatory loss of taurine via the bile salts [22]. They therefore depend, to a large extent, on taurine obtained through diet, which must be transported across several cell membranes to accumulate in the heart [22]. The recent application of molecular biological techniques to the taurine research field led to the isolation and characterization of cDNAs that encode taurine transporters from many tissues, primary cultures, and established cell lines [3–10,23]. There are no known studies showing the details of the taurine transporter in primary cultured cardiac myocytes and nonmyocytes. In the present study, we examined the characteristics of the taurine transporter and the intracellular taurine content in two types of cultured heart cells (myocytes and nonmyocytes).

First, the intracellular taurine content (21 ± 2 nmol/10^6 cells and 181 ± 21 pmol/μg protein) of myocytes was significantly higher than that of nonmyocytes (7 ± 1 nmol/10^6 cells and 84 ± 14 pmol/μg protein). The difference in content between the myocytes and nonmyocytes may relate to function and morphology. Myocytes in culture keep contracting spontaneously, while nonmyocytes lack this characteristic. This may be important since the $[Ca^{2+}]_i$ undergoes dramatic changes during the contraction–relaxation cycle [24]. It is possible that the contracting cell may become more susceptible to the Ca^{2+}-modulating actions of taurine [25–27].

Second, using northern blot analysis we found taurine transporter mRNA strongly expressed in both myocytes and nonmyocytes. The magnitude (normalized to GAPDH gene expression) of the transporter mRNA expressed in myocytes was lower than that in nonmyocytes. The expression of taurine transporter proteins as assessed by western blot analysis was also similar to the mRNA results.

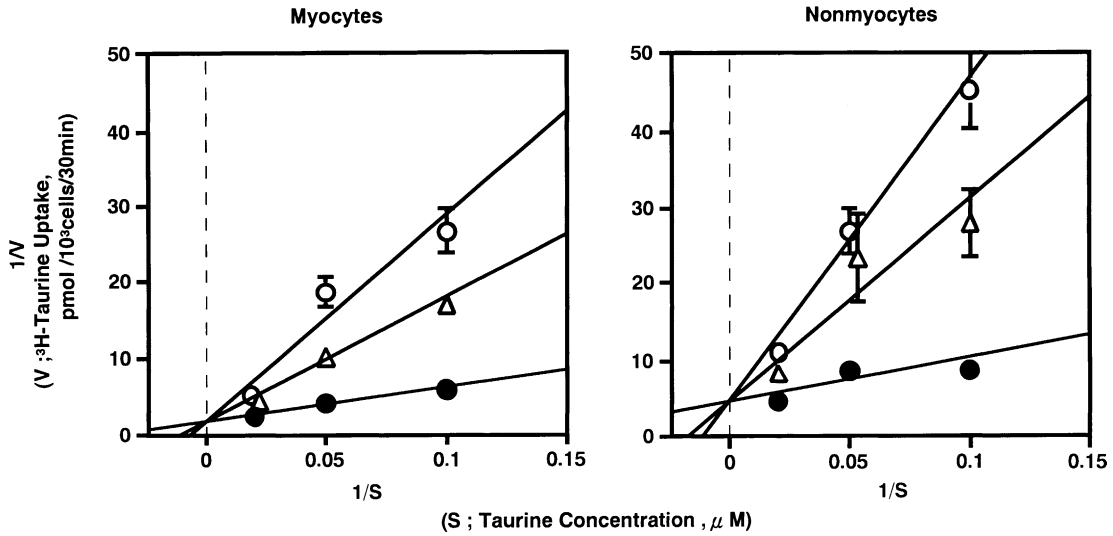

Fig. 4. Lineweaver–Burk plots showing the inhibition by β-alanine of taurine uptake in neonatal rat heart cells. Taurine uptake was measured over a β-alanine concentration range of 0–250 μM (closed circles: 0 μM; open triangles: 100 μM; open circles: 250 μM) for a 30-min incubation. The total concentration of taurine ([^{3}H]taurine (0.5 μCi/mL) and unlabeled taurine) was 10–50 μM. Values are means ± SEM of 12 samples obtained from 3 different cultures.

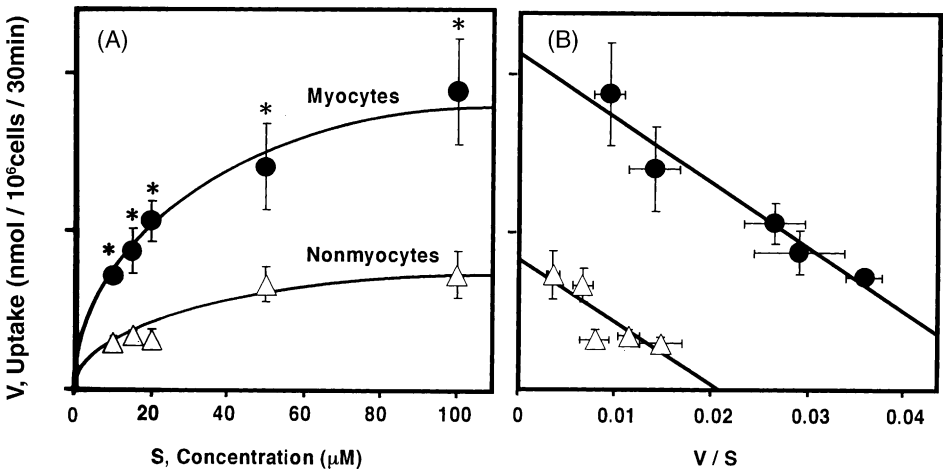

Fig. 5. Kinetic analysis of taurine uptake in neonatal rat heart cells. (A) Taurine uptake was measured in cells incubated for 30 min with buffer containing NaCl. Values are means ± SEM of 6–12 samples obtained from 3 different cultures. Key: (*) $P < 0.01$ versus nonmyocytes at each concentration. (B) Graph shows Eadie–Hofstee plots.

However, the present results also indicated that total protein content per myocyte was significantly higher (1.6-fold) than per nonmyocyte. The possibility that the difference of total protein per cell relates to the content of transporter protein cannot be excluded. This is the first evidence of the presence of the taurine transporter system in cardiac fibroblasts obtained from neonatal rat hearts.

Third, we studied the kinetics of taurine transport using myocytes and nonmyocytes isolated from neonatal rat hearts. The uptake of [^{3}H]taurine into cultured heart cells was Na^+- (Fig. 2) and Cl^--dependent [3] and also was inhibited competitively by another β-amino acid, β-alanine (Fig. 4). Parallel incubations were performed at 4° to correct for errors due to non-specific binding. There was no statistical difference in taurine uptake at 4° with and without exogenous taurine (data not shown). The taurine transporter on heart cells exhibits saturation kinetics. Some studies [7,19,20,28] have shown that human cell lines actively accumulate taurine and that the transport process is Na^+- and temperature-dependent and sensitive to metabolic poisons. Three different driving forces, namely a Na^+ gradient, a Cl^- gradient, and membrane potential, energize the transport system. In several cell lines, such as Caco-2 cells (intestine) and HRPE (retinal pigment epithelium), the Na^+:Cl^-:taurine stoichiometry is 2:1:1 [19,20,29]. From our data, myocytes and nonmyocytes showed sigmoidal sodium dependency with a Hill coefficient of about 2.0, indicating the requirement of two sodium ions for the translocation of one molecule of taurine (Fig. 3). The present findings in cultured heart cells are in good accord with previous observations.

Furthermore, the kinetic analysis showed that a single saturable system, one of high affinity, was involved in taurine uptake over a range of 0–100 μM in both myocytes and nonmyocytes. In myocytes, the apparent K_m and V_{max} for the transporter were 20.7 ± 0.5 μM and 1.07 ± 0.01 nmol/10^6 cells/30 min, respectively. Similarly, those of nonmyocytes were 20.3 ± 0.7 μM and 0.42 ±0.01 nmol/10^6 cells/30 min. It is plausible that the higher V_{max} in myocytes may lead to greater accumulation of taurine. There are also other factors to consider such as the possible existence of storage pools in myocytes. Further studies are needed to fully understand the role of the taurine transporter in the heart. Although plasma levels of taurine are much lower, ranging from 30 to 500 μM depending on the species [2], it should be noted that the K_m values obtained from rat neonatal heart cells are about 20 μM, and these carriers would be operating at their K_m values. There is a significant difference in the affinity of the transporter for taurine in different cell lines. For example, the values for the Michaelis–Menten constant vary from 0.9 μM in Caco-2 cells to 11 μM in HT-29 cells, both human colon carcinoma cell lines [29].

These present findings indicated that both myocytes and nonmyocytes expressed an identical taurine transporter with a Michaelis–Menten constant of 20–21 μM, whereas each cell type had a different activity. Namely, the higher taurine content in myocytes may be associated with a higher V_{max}.

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