ELSEVIER

Biochimica et Biophysica Acta 1371 (1998) 335–344

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Calyculin A modulates the kinetic constants for the Na^+-coupled taurine transport in Ehrlich ascites tumour cells

Jens Mollerup *, Ian Henry Lambert

The August Krogh Institute, Biochemical Department, University of Copenhagen, 13 Universitetsparken, DK-2100 Copenhagen, Denmark

Received 21 November 1997; revised 13 February 1998; accepted 5 March 1998

Abstract

The effect of the phosphatase inhibitor calyculin A (cal A) on the kinetic parameters of the Na^+-coupled taurine uptake via the taurine transporter in the Ehrlich ascites tumour cells has been investigated. Preincubation with cal A (100 nM) reduces the initial taurine influx by about 20%, but has no effect on the diffusional component of the taurine influx or on the taurine release from cells suspended in isotonic or in hypotonic medium. Thus, cal A-sensitive phosphatases only affect taurine transport mediated by the Na^+-dependent taurine transporter. Cal A increases the Michaelis–Menten constant for binding of taurine to the transporter from 31 ± 6 to 45 ± 4 μM and reduces the taurine transport capacity from 210 ± 20 to 170 ± 10 pmol $\times$ g dry wt$^{-1} \times$ min^{-1}. The Michaelis–Menten constant for binding of Na^+ to the taurine transporter is concomitantly increased from 96 ± 11 to 129 ± 8 mM and the Na^+:taurine coupling ratio for activation of the transport cycle is reduced from 3.3 ± 0.6 to 2.4 ± 0.2. This suggests that cal A-sensitive phosphatases maintain a high affinity of the taurine transporter towards Na^+ and taurine as well as a high taurine transport capacity in unpertubated Ehrlich cells.

Keywords: Calyculin A; Phosphatase; Carrier kinetics; Taurine transporter; Ehrlich ascites tumour cell; Na^+ coupling; Taurine channel

1. Introduction

Taurine (2-aminoethanesulfonic acid), a widespread sulfur containing amino acid, is highly concentrated in cells of skeletal and heart muscle and in cells of the mammalian nervous system [1]. In Ehrlich mouse ascites tumour cells, the cellular taurine concentration has been estimated at 53 mM [2]. Taurine has a range of important biological functions including the role of an organic osmolyte [1,3]. In the Ehrlich cells, cell swelling stimulates taurine efflux and the net loss of taurine following a 50% reduction in the extracellular osmolarity constitutes 30% of the total loss of osmolytes [4]. The high intracellular concentration of taurine in Ehrlich cells is maintained by the β-amino acid transporter (β-system, taurine transporter), which has a high affinity for taurine ($K_{tau} \approx 60$ μM) [5] but a low transport capacity ($J_{max} \approx 450$ pmol $\times$ g dry wt$^{-1} \times$ min^{-1}) [5]. Anions are required for taurine influx via the taurine transporter with the following preference $Cl^- > SCN^- > NO_3^-$ [5]. In the Ehrlich cells the initial taurine influx is totally Na^+-dependent and the Na^+:taurine coupling ratio for initiation of taurine influx has been estimated at 2:1 at 100 μM extracellular taurine [5]. From other cell lines a 3 Na^+:1 taurine coupling ratio has been demonstrated, e.g., in rabbit kidney brush

* Corresponding author. Fax: +45-3532-1694; E-mail: jmollerup@aki.ku.dk

0005-2736/98/$19.00
PII S0005-2736(98)00033-9

border membranes [6], in rabbit jejunal brush border membranes [7], and in a human kidney proximal tubule cell line [8].

Taurine uptake in Ehrlich cells is reduced following depolarisation of the cell membrane, acidification of the extracellular medium [9], and following osmotic shrinkage [10]. Phosphorylation of the taurine transporter or a regulator of the transporter by protein kinase C (PKC) has also been shown to reduce the taurine influx in the Ehrlich cells [11], in the human choriocarcinoma cell line (JAR, see Ref. [12]), in human colon cells (HT-29, Caco-2, see Ref. [13]), and in rat astrocytes [14]. In the case of the Ehrlich cells it has been suggested that the taurine transporter appears in three states: *state I*, *state II*, and *state III* exhibiting low, normal, and high transport activity, respectively [11]. Transition from *state II* to *state I* is mediated by PKC, whereas, the reverse transition is mediated by cal A-sensitive phosphatases [11]. Furthermore, transition from *state II* to *state III* requires an elevation of the intracellular cAMP content [11]. The taurine transporter has been suggested to be retained in *state I* following inhibition of cal A-sensitive phosphatases [11].

The present paper focuses on the effect of cal A-sensitive phosphatases on the kinetic parameters of taurine transport in the Ehrlich cells. It is demonstrated that inhibition of dephosphorylation retains the taurine transporter in a state with low transport activity (*state I*) where its apparent affinity towards Na^+ and taurine, its transport capacity, and the coupling ratio between Na^+ and taurine are reduced compared to a state with normal transport activity (*state II*).

2. Materials and methods

2.1. Cell suspension

Ehrlich ascites tumour cells (hyperdiploid strain), grown by weekly intraperitoneal inoculation of female NMRI (Naval Medical Research Institute) mice, were harvested in standard medium containing 2.5 IU $\times$ ml^{-1} heparin. Cells were washed twice in standard medium without heparin by centrifugation (700 $\times g$, 45 s) and finally resuspended in standard medium at a cytocrit of 4–6%. Before any further treatment the cells were incubated for 30 min at 37°C. All experiments were carried out at 37°C.

2.2. Inorganic media

The standard medium contained: 143 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, Na_2HPO_4, and $MgSO_4$, 3.3 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS), *N*-tris-(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) and *N*-(2-hydroxyethyl)-piperazine-*N*′-(2-ethanesulfonic acid) (HEPES). Nagluconate medium was prepared by substitution of gluconate for Cl^-. In media containing between 20–150 mM Na^+, *N*-methyl-D-glucamine chloride (NMDG) was substituted for NaCl. In experiments with increasing extracellular concentrations of taurine (10 μM–2 mM), taurine was added from a stock solution prepared in standard medium. Hypertonicity was induced by addition of one part concentrated sucrose medium (54.6–209.5 g sucrose $\times$ l $medium^{-1}$) to five parts standard medium. Hypotonic medium with half of the original osmolarity was prepared by dilution of the standard medium with buffered water. All solutions used to change the osmolarity of the cell suspension contained the same concentration of buffers as described above. All media were adjusted to pH 7.4 by addition of NaOH at room temperature.

2.3. Estimation of initial taurine influx

The initial taurine influx J_{tau} (pmol $\times$ g dry wt^{-1} $\times$ min^{-1}) was calculated as the rate constant (g medium $\times$ g dry cell wt^{-1} $\times$ min^{-1}) of ^{14}C-taurine influx times the medium taurine concentration (μmol $\times$ g $medium^{-1}$ as previously described [11]). In brief, ^{14}C-taurine 0.16 μCi $\times$ ml^{-1} was added to the cell suspension at time zero and five samples (1000 μl) of the cell suspension were drawn with 1 min intervals. The samples were centrifuged (15 000 $\times g$, 45 s) to separate cells and extracellular medium. Wet weight was estimated, the cell pellet dissolved in water, the medium diluted with water, and the proteins precipitated by addition of perchloric acid (final concentration 7%). The dry weight was estimated from the perchloric acid precipitate, which constitutes the cell dry weight $\times$ 1.30 [15]. The ^{14}C-taurine activity was estimated by scintillation counting. Cellular ^{14}C-taurine activity was calculated by subtracting ex-

tracellular trapped ^{14}C-taurine activity according to the method of Hoffmann et al. [16] using ^{3}H-PEG instead of ^{3}H-inulin as the extracellular marker. The rate constant of the initial ^{14}C-taurine influx was calculated as the slope of $a_c^t \times (a_m^0)^{-1}$ plotted vs. time, where a_c^t is the cellular ^{14}C-taurine activity at time t (DPM × g dry wt^{-1}) and a_m^0 is the ^{14}C-taurine activity in the medium at time zero (DPM × ml^{-1}) as estimated by extrapolation of the amount of ^{14}C-taurine activity in the extracellular medium at the five time points.

A variation of the taurine influx experiment described above was used in experiments with reduced extracellular Na^+ concentrations. In this case the cells were washed by centrifugation (700 × g, 45 s), and resuspended in the medium with the desired Na^+ concentration just before initiation of the influx experiment. ^{14}C-taurine (0.16 μCi × ml^{-1}) was added to the cell suspension at time zero and duplicate samples were drawn at 5 min. ^{14}C-taurine and ^{3}H-PEG activity was correlated with cells and extracellular media as indicated above. The initial taurine influx J_{tau}, which is linear within the first 5 min [11], was calculated as $a_c^5 \times (a_m^0)^{-1} \times [tau] \times 5^{-1}$, where a_c^5 is the ^{14}C-taurine activity in the cells at 5 min (DPM × g dry wt^{-1} × 5 min), a_m^0 is the ^{14}C-taurine activity^{-1} (DPM × ml^{-1}) in the extracellular medium at time zero and [tau] is the initial taurine concentration (μmol × ml medium^{-1}) in the extracellular medium. To estimate the initial taurine influx at different taurine concentrations cells suspended in standard medium were directly transferred to new flasks containing the ^{14}C-taurine (0.16 μCi × ml^{-1}) plus unlabelled taurine. Sampling was carried out as described for experiments with varying extracellular Na^+ concentrations.

2.4. Estimation of initial taurine efflux

The initial taurine efflux from Ehrlich cells was estimated as described by Lambert and Hoffmann [17]. In brief, Ehrlich cells (cytocrit 6%) were loaded with ^{14}C-taurine (0.5 μCi × ml cell suspension^{-1}) for 60 min. For initiation of the efflux experiment, 1 ml of the cell suspension was gently packed by centrifugation (770 × g, 1 min) in a nylon tube (i.d. 3 mm). The part of the tube containing the packed cells was cut free and the cells were flushed into 8 ml of Nagluconate efflux medium (time zero). Seven samples were taken within the first minute for estimation of ^{14}C-taurine activity in the extracellular medium. The samples were centrifuged through silicone oil (1 part 200 DC20 to 10 parts AR 200) to separate the cells and the extracellular medium. At the end of the efflux experiment double samples were taken from the efflux flask to estimate the protein content using BSA standards [18]. The rate constant for the initial taurine efflux (ml cell water × g dry wt^{-1} × min^{-1}) was estimated as the slope of the plot $(a_m^t - a_m^0) \times (a_c^0)^{-1}$ vs. time (see Fig. 3) multiplied by the cell water content (ml cell water × g dry wt^{-1}), where a_m^t and a_m^0 are the ^{14}C-taurine activity (DPM × ml medium^{-1}) of the extracellular medium at time t and zero, respectively, and a_c^0 is the intracellular ^{14}C-taurine activity (DPM × g dry wt^{-1}) at time zero.

2.5. Estimation of the cell volume, Na^+ and ^{14}C-taurine gradient

The cell volume was estimated as the mean water content (ml cell water × g dry wt^{-1}), corrected for extracellular trapped medium (see above), during the 5-min influx experiments. The concentration of Na^+ [Na^+] was estimated from duplicate samples of the media and the cells after precipitation with PCA (see above), using a Perkin-Elmer 2380 atomic absorption spectrophotometer. The intracellular Na^+ concentration was estimated as mmol Na^+ × l cell water^{-1} after correction for extracellular trapped Na^+. The Na^+ gradients were calculated as the extracellular Na^+ concentration (mmol × l medium^{-1}) divided by the intracellular Na^+ concentration (mmol × l cell water^{-1}). For estimation of the ^{14}C-taurine gradient, ^{14}C-taurine (0.16 μCi × ml medium^{-1}) was added to the cell suspension at time zero, and duplicate samples taken at 10, 20, 30, and 60 min. The ^{14}C-taurine gradient at time t was calculated as the intracellular ^{14}C-taurine activity (a_c^t, DPM × ml cell water^{-1}) divided by the extracellular ^{14}C-taurine activity (a_m^t, DPM × ml medium^{-1}).

2.6. Estimation of the cellular taurine concentration by high pressure liquid chromatography

The taurine content in the Ehrlich cells was estimated by a standard OPA derivatisation procedure

followed by HPLC separation [19]. The cellular concentration of taurine was calculated from the taurine content (μmol $\times$ g dry wt^{-1}) and the water content (ml $\times$ g dry wt^{-1}).

2.7. Chemicals

Cal A (Alamone, Jerusalem, Israel) was prepared in 96% ethanol (20 μM) and kept under a nitrogen atmosphere at -20°C. ^{14}C-taurine and ^{3}H-polyethyleneglycol (DuPont, NEN, Kastrup, Denmark) were diluted in influx media just before use. NMDG, MOPS, TES, HEPES, *ortho*-phtaldehyde (OPA), bovine serum albumin were obtained from Sigma (St. Louis, MO). Sucrose was from BDH (Poole, England). Silicone oil 200 DC 20 (Bie and Berntsen, Copenhagen, Denmark) and AR 200 (Serva, Heidelberg, Germany) were mixed 1:10 (w/w) and stored at room temperature.

2.8. Equations

The maximal transport rate of initial taurine transport (J_{max}), the Michaelis–Menten constant for the apparent taurine binding (K_{tau}) to the taurine transporter as well as the rate constant for the non-saturable influx (k_{diff}) were calculated according to Eq. (1).

$$J_i = \frac{J_{max} \cdot [tau]}{K_{tau} + [tau]} + k_{diff} \cdot [tau] \quad (1)$$

Eq. (2) is a Hill type equation and according to Turner [20], an approximation of the numbers (n) of essential cooperative activator binding sites per substrate transport can be found by fitting the influx data to this equation. K_{Na}, is the Michaelis–Menten constant for the apparent binding of Na^+ to the taurine transporter and J_p is the maximal taurine transport rate at an extracellular taurine concentration [tau] of 10 μM.

$$J_i = \frac{J_p \cdot [Na^+]^n}{(K_{Na})^n + [Na^+]^n} \quad (2)$$

It is emphasised that this method only gives an estimate of the stoichiometric coupling of Na^+ to taurine since it does not distinguish between catalytic and energetic activation [20], i.e., it only describes the number of Na^+ needed for initiation of one transport cycle, whereas, the number of Na^+ cotransported with taurine could be different.

2.9. Statistics

All results are given as mean $\pm$ SEM of at least three independent experiments. When *t*-tests were performed, the data fulfilled the criteria of no difference in variance and the data were normal distributed. Unless otherwise stated a two-tailed significance level of 0.05 was used.

3. Results

3.1. Effect of calyculin A on the initial taurine influx and on the cell volume of Ehrlich cells

Cal A inhibits protein phosphatases of type PP1, PP2A and PP3 [21], and we have previously shown that exposure to cal A significantly reduces the initial taurine influx in Ehrlich cells [11]. This is confirmed in Fig. 1A, where it is seen that addition of cal A (100 nM) to cells suspended in isotonic medium significantly reduces the initial taurine influx by approximately 20%. From Fig. 1B, it is seen that cal A treatment of the Ehrlich cells suspended in isotonic medium also leads to a significant cell swelling. However, if the Ehrlich cells are suspended in hypertonic medium with a high concentration of sucrose (34.9 g $\times$ l^{-1}), which in itself reduces the initial taurine uptake, then addition of cal A (100 nM) has no effect on neither the initial taurine influx (Fig. 1A) nor the cell volume (Fig. 1B). To test whether the effect of cal A on the initial taurine influx under isotonic conditions is secondary to the concomitant cell swelling, we adjusted the cell volume in the cal A-treated cells by adding a small amount of sucrose to the extracellular medium. In Table 1, it is seen that addition of cal A (100 nM) to Ehrlich cells suspended in a hypertonic medium with a low concentration of sucrose (9.1 g $\times$ l^{-1}) results in a cell volume that is just below the cell volume of cells suspended in isotonic medium and a concomitant reduction in the initial taurine influx. Since there is a linear correlation between cell shrinkage in hypertonic medium

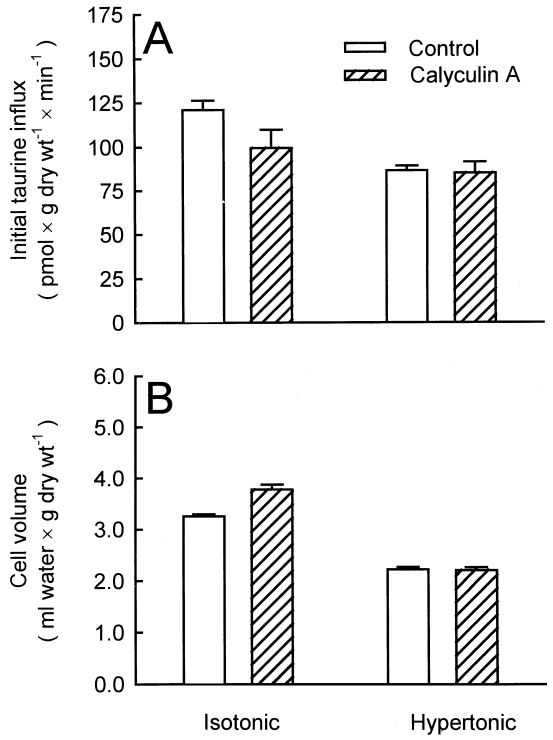

Fig. 1. Effect of cal A on the initial taurine influx (panel A) and cell volume (panel B) in Ehrlich cells suspended in isotonic and in hypertonic medium. Cells were incubated for 0.5 min with cal A (100 nM, hatched bars) before dilution with hypertonic sucrose medium (final concentration 34.9 g×l medium^{-1}) or isotonic medium. Data are from at least three independent experiments, and given as means ± SEM. The effect of cal A on the initial taurine influx and on the cell volume is significant in isotonic medium.

and the reduction in the initial taurine influx [10], we plotted the cell volumes vs. the flux values of cells suspended in isotonic and hypertonic media with no cal A. Using this curve and the cell volume of the sucrose plus cal A-treated cells we estimated a theoretical value of the initial taurine influx. The theoretical value for the initial taurine influx in cells having a cell volume of 3.65 ml water × mg dry wt^{-1} is 90 ± 6 pmol × g dry wt^{-1} × min^{-1} (see legend to Table 1). This value is significantly larger than the experimentally estimated value, i.e., 79 ± 5 pmol × g dry wt^{-1} × min^{-1} (Table 1), indicating that the cal A-induced inhibition of the initial taurine influx in Ehrlich cells is not secondary to the cal A-induced cell swelling.

3.2. Effect of calyculin A treatment on the gradients of Na^+ and ^{14}C-taurine in Ehrlich cells

The cal A-induced inhibition of the initial taurine influx could be secondary to a reduction of the Na^+ gradient, i.e., the driving force for the Na^+-coupled taurine uptake. From Fig. 2A, it is seen that treatment of Ehrlich cells with cal A (100 nM) significantly increases the gradient for Na^+, and that the effect of cal A on the Na^+ gradient is evident at the time of the first sample (10 min) and does not change within the 60-min experimental period. Fig. 2B shows that in the control cells the ^{14}C-taurine gradient increases

Table 1
Effect of cal A on the initial taurine influx in Ehrlich cells with cell volume adjusted close to isotonic cell volume by addition of a low dose of sucrose

	Cell volume (ml × mg dry wt^{-1})	Initial taurine influx (pmol × g dry wt^{-1} × min^{-1})
Isotonic	3.85 ± 0.02	103 ± 5
Low hypertonic	3.47 ± 0.03 (0.001)	80 ± 6 (0.009)
Low hypertonic + cal A	3.65 ± 0.03 (0.04)	79 ± 5 (0.04)

Cell volume and initial taurine influx were estimated as described in Section 2. Cal A (100 nM) and/or sucrose (final concentration 9.1 g sucrose × l medium^{-1}) were added to the cell suspension 1.5 min and 1 min before initiation of the taurine influx experiment, respectively. Due to the linearity between cell volume and initial taurine influx [10], it is possible to calculate the initial taurine influx corresponding to a certain cell volume. The mean ± SEM from three independent experiments of the initial taurine influx, corresponding to the cell volume of sucrose plus cal A-treated cells, i.e., 3.65 ml water × g dry wt^{-1}, is 90 ± 6 pmol × g dry wt^{-1} × min^{-1} as estimated from the cell volume and the initial taurine influx in the control cells and in cells treated with sucrose. The difference in influx between the calculated, theoretical value, and the observed value, calculated from each set of experiments and tested against zero difference (i.e., no effect of cal A) is significant ($P = 0.007$, one sample, two-tailed *t*-test). The data in the table are taken from five different experiments (three independent sets of experiments). *P*-values (different from isotonic) are indicated in the parentheses.

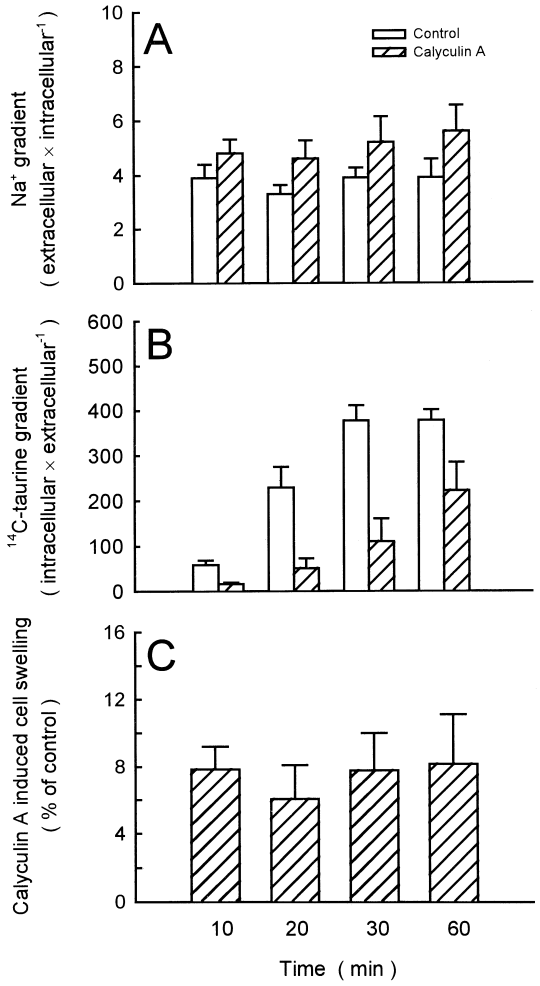

Fig. 2. The effect of cal A on the Na^+ gradient (panel A), the ^{14}C-taurine gradient (panel B), and the cell volume (panel C). Ehrlich cells were incubated in standard medium in the absence (open bars) or presence of cal A (100 nM, hatched bars). Duplicate samples were taken at 10, 20, 30 and 60 min. Data from panels A, B and C are from the same four independent experiments. The Na^+ gradient is given as the extracellular concentration divided by the intracellular concentration. Values within the control series and within the cal A-treated series do not significantly differ ($P > 0.5$). The overall mean $\pm$ SEM of the Na^+ gradient at 10, 20, 30, 60 min in control cells (3.8 ± 0.2) and in cal A-treated cells (5.1 ± 0.2) are significant different ($P = 0.004$). The taurine gradient was estimated as the intracellular concentration of ^{14}C-taurine (DPM $\times$ ml cell water^{-1}) divided by the extracellular concentration (DPM $\times$ ml medium^{-1}). The ^{14}C-taurine gradient was significantly reduced by cal A at time 10, 20, and 30 min ($P < 0.02$), and significantly reduced at 60 min ($P = 0.04$, one sided, unpaired *t*-test). The cal A-induced cell swelling is given as the increase in cell volume in percent of the cell volume of control cells. The cal A-induced cell swelling is significant larger than zero ($P = 0.005$).

rapidly, reaching a plateau close to 400 at about 30 min following the addition of the isotope, whereas the ^{14}C-taurine accumulation is severely reduced in the presence of cal A and no plateau is reached within the 60-min experimental period. Cell swelling leads in itself to a reduction in the intracellular Na^+ concentration and thereby an increased Na^+ gradient. However, as seen from Fig. 2C, the cal A-induced cell swelling is of a magnitude of 8% during the 60-min time course, which does not explain the 35% (legends to Fig. 2A) increase in the Na^+ gradient. Thus, although the Na^+ gradient is enhanced in cal A-treated cells the efficiency by which the cells build up a ^{14}C-taurine gradient is impaired. It is noted that the cal A-induced cell swelling (Fig. 1B) most probably is due to activation of the Na^+, K^+, $2Cl^-$ cotransporter which promotes an uptake of salt and water [22]. Since Na^+ taken up by the Na^+, K^+, $2Cl^-$ cotransporter recycles through the Na^+, K^+ pump [23] it is likely that the activation of the Na^+, K^+, $2Cl^-$ cotransporter by cal A results in stimulation of the Na^+, K^+ pump which could lead to the steeper Na^+ gradient (Fig. 2A).

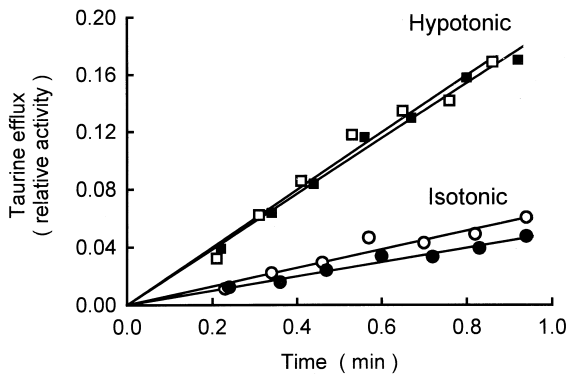

Fig. 3. Effect of cal A on the initial taurine efflux from Ehrlich cells in isotonic (circles) and hypotonic (squares) medium. Cal A (100 nM, closed symbols) was added 2 min before initiation of the efflux experiment. Taurine efflux is given as the difference between the ^{14}C-taurine activity of the extracellular medium at time *t* and zero divided by the intracellular ^{14}C-taurine activity at time zero (see Section 2). The rate constants (ml cell water $\times$ g dry wt^{-1} $\times$ min^{-1}) were estimated at 0.18 ± 0.04 (isotonic control), 0.18 ± 0.01 (isotonic with cal A), 1.46 ± 0.09 (hypotonic, control) and 1.24 ± 0.03 (hypotonic with cal A). Cal A has no significant effect on the rate constant for taurine efflux from cells in isotonic medium ($P = 1.0$) or in hypotonic medium ($P = 0.2$) in a two-tailed, paired *t*-test (three independent experiments).

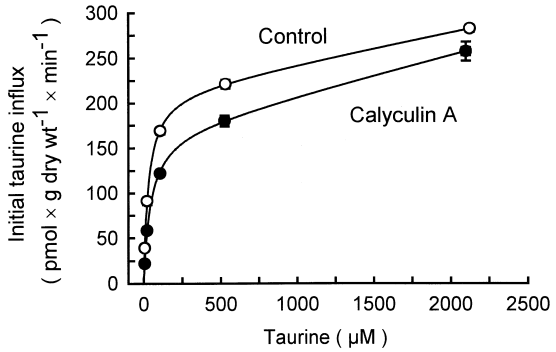

Fig. 4. Effect of cal A on the initial taurine influx in Ehrlich cells at increasing extracellular taurine concentrations. The extracellular Na^+ concentration was 150 mM and the extracellular taurine concentration varied between 5 and 2100 μM. Cal A (100 nM, closed circles) was added 1.5 min before initiation of the taurine influx experiment. Data from three independent experiments are plotted as means ± SEM and fitted to Eq. (1). If not visible, the error bars are within the symbol. The lines representing the best fit to Eq. (1) are drawn. J_{max}, K_{tau} and k_{diff} values are given in Table 2.

3.3. Calyculin A has no effect on taurine release

To delineate if the diminished ^{14}C-taurine gradient induced by cal A is secondary to an increased taurine permeability, we estimated the rate constant for ^{14}C-taurine efflux and the cellular taurine concentration in Ehrlich cells preincubated with cal A as well as in control cells. In Fig. 3, it is confirmed that the initial taurine efflux from Ehrlich cells is increased following transfer to a hypotonic medium. However, preincubation with cal A (100 nM) does not affect the release of ^{14}C-taurine from cells suspended neither in the isotonic nor in the hypotonic media. The intracellular concentration of taurine in Ehrlich cells, estimated by HPLC, is 40 ± 1.2 mM in control cells and 36 ± 1.4 mM in cells preincubated for 60 min with cal A (100 nM) (values are not significant different, two-tailed, paired *t*-test, $n = 4$). Thus, cal A has no effect on the steady state taurine gradient from Ehrlich cells, indicating that the effect of cal A on the ^{14}C-taurine gradient is not secondary to a change in the taurine permeability. It should be noticed that even though cal A leads to a persistent cell swelling (Fig. 2C) it does not stimulate the taurine release, indicating that the taurine channel normally activated by hypotonic cell swelling [17] is not activated under isotonic conditions by the cal A-induced cell swelling.

3.4. Effect of calyculin A on the Na^+ to taurine coupling ratio and the kinetic parameters of taurine transport

The previous data indicate that cal A treatment diminishes the efficiency of the taurine transporter in the Ehrlich cells. To understand the mechanism whereby cal A modifies the taurine transport in Ehrlich cells we estimated the taurine transport capacity of the taurine transporter, the Michaelis–Menten constants for taurine and for Na^+ binding to the taurine transporter as well as the Na^+:taurine coupling ratio. Fig. 4 shows the initial taurine influx at increasing concentrations of extracellular taurine in the presence and in the absence of cal A (100 nM). The best fit of these data was obtained by fitting each experiment to the sum of a saturable influx component and a non-saturable influx component (Eq. (1), Section 2). For cells suspended in isotonic medium J_{max}, K_{tau} and k_{diff} were estimated at 210 ± 20 pmol × g dry wt^{-1} × min^{-1}, 31 ± 6 μM and 0.03 ± 0.01 ml × g dry wt^{-1} × min^{-1}, respectively (Table 2). These values are comparable to previously reported

Table 2
Effect of cal A on the kinetic parameters of initial taurine influx in Ehrlich cells at 150 mM NaCl

	J_{max} (pmol × g dry wt^{-1} × min^{-1})	K_{tau} (μM)	k_{diff} (ml × g dry wt^{-1} × min^{-1})
Control	210 ± 20	31 ± 6	0.03 ± 0.01
Cal A	170 ± 10 (0.03)	45 ± 4 (0.005)	0.04 ± 0.01

The influx data from Fig. 4 are fitted to the sum of one saturable and one non-saturable component of initial taurine influx (see Eq. (1)). The means of the maximal initial taurine influx (J_{max}) and Michaelis–Menten constant (K_{tau}), as well as the rate constant for the non-saturable component (k_{diff}) are given ± SEM from three independent sets of experiments. *P*-values (difference from control) are indicated in the parentheses.

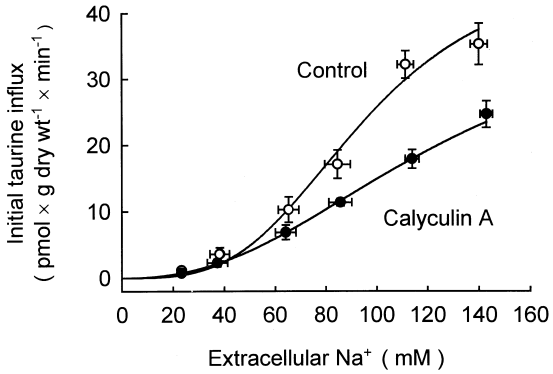

Fig. 5. Effect of cal A on the initial taurine influx in Ehrlich cells at increasing extracellular Na^+ concentrations. The extracellular taurine concentration was 10 μM. The extracellular Na^+ concentrations were varied between 0 and 150 mM by substituting $NMDG^+$ for Na^+. Cal A (100 nM, closed circles) was added 1.5 min before initiation of the taurine influx experiment. The estimated Na^+ concentrations and the resulting initial taurine influx of four independent experiments are plotted as means ± SEM, and fitted to Eq. (2). The lines represent the best fit to Eq. (2). K_{Na} and the Na^+:taurine coupling ratio are given in Table 3.

kinetic constants for taurine transport from Ehrlich cells [5]. Treatment of the Ehrlich cells with cal A (100 nM) significantly reduces J_{max} and increases K_{tau} of the initial taurine influx, whereas it has no effect on the taurine diffusion k_{diff} (Table 2). This indicates that inhibition of cal A-sensitive phosphatases leads to a reduction in the apparent affinity of the taurine transporter to taurine and in the taurine transport capacity.

Table 3
Effect of cal A on the Na^+:taurine coupling ratio and on the Michaelis–Menten constant for Na^+ binding to the taurine transporter at 10 μM extracellular taurine in Ehrlich cells

	K_{Na} (mM)	Na^+:taurine coupling ratio
Control	96 ± 11	3.3 ± 0.6
Cal A	129 ± 8 (0.02)	2.4 ± 0.2 (0.04)

Influx data from each of four independent experiments, plotted in Fig. 5, were fitted to Eq. (2) to give the Na^+:taurine coupling ratio, i.e., the number of Na^+ needed for initiation of the taurine transport cycle, as well as the Michaelis–Menten constant (K_{Na}) for Na^+ binding. Data are given as means ± SEM. *P*-values (difference from control) are indicated in the parentheses from a paired (K_{Na}) or unpaired (coupling ratio), two-tailed *t*-test. The maximal transport rate at 10 μM taurine (J_p), estimated at 50 ± 20 and 40 ± 10 pmol × g dry $wt^{-1} \times min^{-1}$ in control cells and cal A (100 nM)-treated cells, respectively, was not significantly different.

Fig. 5 shows the correlation between the initial taurine influx and the extracellular Na^+ concentration at 10 μM taurine in the presence and in the absence of cal A (100 nM). The sigmoid pattern confirms previous data [5] which indicated that more than one Na^+ binds to the taurine transporter during initiation of the taurine transport cycle. The present data were fitted to Eq. (2), (see Section 2), and the means ± SEM of the coupling ratio between Na^+ and taurine and the apparent affinity of the taurine transporter for Na^+ are given in Table 3. As seen from Table 3, cal A treatment significantly reduces the Na^+:taurine coupling ratio from 3.3 to 2.4 and the apparent affinity of the taurine transporter for Na^+ (K_{Na} increased from 96 to 129 mM). Thus, the cal A-sensitive phosphatases in Ehrlich cells only seem to affect transport kinetics for taurine uptake via the taurine transporter, whereas they have no effect on passive taurine influx or efflux.

4. Discussion

Regulation of membrane transport pathways has in many cases been shown to involve phosphorylation and dephosphorylation. However, at the functional and molecular level it is almost unknown how covalent modification changes the kinetic parameters of amino acid transport. A reduction in the maximal taurine transport rate and a decrease in the affinity of the taurine transporter for taurine following activation of PKC have been demonstrated in human colon cells (HT-29 and Caco-2) [13] and in rat astrocytes [14]. Furthermore, it has been shown that the taurine transporter (pNCT) isolated from MDCK cells and expressed in *Xenopus laevis* oocytes has a putative PKC phosphorylation site at an intracellular segment that seems to be continuously phosphorylated under isotonic conditions [24]. Masking this putative PKC phosphorylation site, by microinjection of a polyclonal peptide antibody, stimulates taurine influx in the *Xenopus laevis* oocyte [24]. In the Ehrlich cells, it has also been demonstrated that PKC mediated phosphorylation besides reducing the transport activity renders the taurine transporter insensitive to stimulation by cAMP [11].

4.1. Calyculin A decreases the Na^+:taurine coupling ratio and the apparent affinity of the taurine transporter to Na^+ and taurine

The data presented in this paper demonstrates that inhibition of the cal A-sensitive phosphatases in the Ehrlich cells reduces active taurine transport, whereas it has no effect on the passive taurine transport, i.e., taurine efflux (Fig. 3) and the non-saturable influx component (Table 2). The reduction in active taurine transport by cal A is not secondary to the concomitant cell swelling (Table 1), and since cal A does not reduce the Na^+ gradient (Fig. 2A) or affect the cell membrane potential [11], it is suggested that the effect of cal A does not reflect a reduced driving force for the Na^+-coupled taurine uptake. The ^{14}C-taurine gradient in cal A-treated cells has the energetic potential to exceed 5000 as calculated from estimated values of cellular and extracellular Na^+ and Cl^- concentrations, a membrane potential of -63 mV [11], and a 2.4 Na^+:1 Cl^-:1 taurine coupling ratio (Table 3, [25]). However, in the presence of cal A, the ^{14}C-taurine gradient only approximates 250 during the experimental period (60 min, Fig. 2B), indicating that cal A affects the initiation of the taurine transport cycle. From Table 3, it is seen that cal A reduces the apparent affinity of the taurine transporter to Na^+ and taurine, as well as the Na^+:taurine coupling ratio for activation of the transport cycle. Previously, it has been demonstrated that binding of Na^+ to the taurine transporter in Ehrlich cells is a prerequisite for taurine uptake, i.e., Na^+ binds before taurine to the transporter and no taurine is taken up in the absence of extracellular Na^+ [5]. Since Na^+ augments the affinity of the taurine transporter for taurine [5], it is likely that the cal A-induced reduction in the taurine affinity (Table 2) is secondary to a reduced binding of Na^+ to the taurine transporter. From Fig. 5 and Table 3 it is deduced that the number of Na^+ needed for initiation of the transport cycle is 3.3 and that this number of Na^+ is reduced to 2.4 in cal A-treated cells. It is emphasised that the kinetic approach does not distinguish between catalytic and energetic activation [20]. If the carrier transports multiple Na^+ (Table 3) and one Cl^- [25] the taurine uptake process is expected to be electrogenic. Electrogenic taurine transport has been demonstrated in *Xenopus laevis* oocytes expressing high levels of the Na^+–taurine cotransport protein [26]. Assuming a membrane capacitance of 1 μF, a membrane surface area of 59 000 $cm^2 \times g$ dry wt^{-1} of the Ehrlich cells [27] and a 3 Na^+:1 Cl^-:1 taurine coupling ratio, it is estimated that Ehrlich cells at the maximal transport rate at 10 μM extracellular taurine (50 pmol $\times$ g dry $wt^{-1} \times min^{-1}$, legend to Table 3) should depolarise the Ehrlich cells by 164 mV $\times$ min^{-1}. However, no change in the membrane potential, as estimated by the fluorescent dye $diOC_3$-(5), could be detected in the Ehrlich cells during taurine uptake [9]. This could indicate that positive charge (e.g., K^+ or Na^+) is returned to the extracellular compartment during the transport cycle, or that Na^+ recycles via the Na^+, K^+ pump at an increased rate.

4.2. Regulation of the taurine cotransporter

According to the three state model for the regulation of the taurine transporter in the Ehrlich cells (see Section 1) the taurine transporter is retained in state I, following exposure to cal A. In state I the transport activity is reduced by 20% compared to state II (Fig. 1), and it can no longer be stimulated by cAMP/PKA [11]. These data could indicate that the taurine transporter might be present in two or more isoforms with the dominating isoform being insensitive to cAMP/PKA and cal A. However, this would imply that the activity of the cAMP/PKA regulated isoform should be 4-fold activated in order to account for the observed 60% increase in taurine influx following activation by dibutyryl-cAMP [11]. Alternatively, the Ehrlich cells only have one isoform of the taurine transporter which is regulated by several signalling pathways, one of which involves change in the phosphorylation level of an activator or an inhibitor of the taurine transporter by cAMP/PKA and cal A-sensitive phosphatases.

Since the Na^+, K^+, 2 Cl^- cotransporter in the Ehrlich cells is activated by osmotic cell shrinkage [23] and by cal A [22], whereas the taurine transporter is inhibited under the same conditions, it is possible that these transporters are regulated in an opposite manner by the same signalling pathways. The activity of PKC is increased following cell shrinkage [28]. However, the shrinkage-induced reduction in the initial taurine influx in Ehrlich cells is larger in the presence of the PKC inhibitor chelerythrine [10],

indicating that shrinkage-induced inhibition of the initial taurine influx does not involve PKC mediated phosphorylation. Whether cell shrinkage in the Ehrlich cells leads to inhibition of cal A-sensitive phosphatases needs further investigation.

Acknowledgements

This work has been supported by the Danish Research Council and by the NOVO Foundation. Dr. John Mills is acknowledged for reading the manuscript and Karen Dissing for technical assistance.

References

[1] R.J. Huxtable, Physiol. Rev. 72 (1) (1992) 101–163.
[2] E.K. Hoffmann, I.H. Lambert, J. Physiol. 338 (1983) 613–625.
[3] R.J. Huxtable, Prog. Neurobiol. 32 (1989) 471–533.
[4] K.B. Hendil, E.K. Hoffmann, J. Cell. Physiol. 1 (1974) 115–125.
[5] I.H. Lambert, Mol. Phys. 6 (1984) 233–246.
[6] N.A. Wolff, R. Kinne, J. Membr. Biol. 102 (1988) 131–139.
[7] Y. Miyamoto, C. Tiruppathi, V. Ganapathy, F.H. Leibach, Am. J. Physiol. 257 (1989) G65–G72.
[8] H. Jessen, Biochim. Biophys. Acta 1 (1994) 44–52.
[9] I.H. Lambert, E.K. Hoffmann, J. Membr. Biol. 131 (1993) 67–79.
[10] J. Mollerup, I.H. Lambert, Acta. Physiol. Scand., 1997, Abstract, in press.
[11] J. Mollerup, I.H. Lambert, J. Membr. Biol. 150 (1996) 73–82.
[12] P. Kulanthaivel, D.R. Cool, S. Ramamoorthy, V.B. Mahesh, F.H. Leibach, V. Ganapathy, Biochem. J. 277 (1991) 53–58.
[13] M. Brandsch, Y. Miyamoto, V. Ganapathy, F.H. Leibach, Am. J. Physiol. 264 (1993) G939–G946.
[14] G.C. Tchoumkeunzouessa, G. Rebel, Am. J. Physiol. 270 (1996) C1022–C1028.
[15] I.H. Lambert, E.K. Hoffmann, F. Jørgensen, J. Membr. Biol. 111 (1989) 113–132.
[16] E.K. Hoffmann, L.O. Simonsen, C. Sjøholm, J. Physiol. 296 (1979) 61–84.
[17] I.H. Lambert, E.K. Hoffmann, J. Membr. Biol. 142 (1994) 289–298.
[18] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, J. Biol. Chem. 193 (1951) 265–275.
[19] B. Kramhøft, I.H. Lambert, Amino Acids 12 (1997) 57–75.
[20] R.J. Turner, J. Membr. Biol. 76 (1983) 1–15.
[21] R.E. Honkanen, B.A. Codispoti, K. Tse, A.L. Boynton, Toxicon 3 (1994) 339–350.
[22] T. Krarup, L.D. Jakobsen, B.S. Jensen, E.K. Hoffmann, Am. J. Physiol., 1998, in press.
[23] E.K. Hoffmann, C. Sjøholm, L.O. Simonsen, J. Membr. Biol. 76 (1983) 269–280.
[24] X.B. Han, A.M. Budreau, R.W. Chesney, J. Am. Soc. Nephrol. 7 (1996) 2088–2096.
[25] I.H. Lambert, Mol. Phys. 7 (1985) 323–332.
[26] D.D.F. Loo, J.R. Hirsch, H.K. Sarkar, E.M. Wright, FEBS Lett. 392 (1996) 250–254.
[27] E.K. Hoffmann, I.H. Lambert, L.O. Simonsen, J. Membr. Biol. 91 (1986) 227–244.
[28] A.K. Larsen, B.S. Jensen, E.K. Hoffmann, Biochim. Biophys. Acta 1222 (1994) 477–482.