J. Membrane Biol. 150, 73–82 (1996)

The Journal of
Membrane
Biology

Phosphorylation is Involved in the Regulation of the Taurine Influx Via the β-system in Ehrlich Ascites Tumor Cells

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Received: 8 September/Revised: 9 November 1995

Abstract. The role of 3′,5′-cyclic adenosine monophosphate (cAMP), protein kinase A (PKA), protein kinase C (PKC) and phosphatases in the regulation of the taurine influx via the β-system in Ehrlich ascites tumor cells has been investigated. The taurine uptake by the β-system in Ehrlich cells is inhibited when PKC is activated by phorbol 12-myristate 13-acetate (PMA) and when protein phosphatases are inhibited by calyculin A (CLA). On the other hand, taurine uptake by the β-system is stimulated by an increased level of cAMP or following addition of N^6,2′-O-dibutyryl-3′,5′-cyclic adenosine monophosphate (dbcAMP). The effect of dbcAMP is partially blocked by addition of the protein kinase inhibitor H-89, and suppressed in the presence of CLA. It is proposed that the β-system in the Ehrlich cells exists in three states of activity: *State I*, where a PKC phosphorylation site on the transporter or on a regulator is phosphorylated and transport activity is low. *State II*, where the PKC phosphorylation site is dephosphorylated and transport activity is normal. *State III*, representing a state with high transport activity, induced by an elevated cellular cAMP level. Apparently, cAMP preferentially stimulates taurine transport when the β-system is in *State II*.

Key words: Taurine transporter — cAMP — Protein kinase A — Protein kinase C — Protein phosphatases — Calyculin A

Introduction

Taurine, which is a biochemically inert β-amino sulfonic acid (2-amino ethane sulfonic acid), has been ascribed an important role as an organic osmolyte in the adjustment of the cell volume of various invertebrate and vertebrate cells (*see* Huxtable, 1992). The cellular taurine concentration in Ehrlich ascites tumor cells has been estimated at 53 mM (Hoffmann & Lambert, 1983). It is the saturable, high affinity, Na^+- and Cl^--dependent β-system, that accounts for the taurine accumulation in these cells (Lambert, 1984, 1985). The swelling-activated taurine channel, on the other hand, accounts for the net loss of cellular taurine following hypotonic cell swelling (Hoffmann & Lambert, 1983; Lambert & Hoffmann, 1994). It has been estimated that taurine accounts for about 30% of the total net loss of osmolytes during the regulatory volume decrease that follows hypotonic cell swelling of the Ehrlich cells (Hoffmann & Hendil, 1976). Thus, interference with the β-system will affect the accumulation of taurine in the Ehrlich cells and subsequently their cell volume.

Kromphardt demonstrated in 1965 that an acidic site ($pK_A = 6.3$), attached to the β-system in the Ehrlich cells, was essential to the accumulation of taurine, and that the site seemed not to participate directly in the binding of taurine (Kromphardt, 1965). Later it was demonstrated that binding of Na^+ to the β-system was a prerequisite for binding of taurine to the carrier, and that no taurine was taken up in the Ehrlich cells in the absence of extracellular Na^+ (Lambert, 1984). More recently the isoelectric point of a taurine/β-alanine transporter, cloned from a mouse brain cDNA library, has been estimated at 5.98 (Liu et al., 1992). Thus, an increase in pH would increase the amount of the taurine carrier on the anionic form. The taurine influx via the β-system in Ehrlich cells is not accompanied by any measurable change in the cell membrane potential, although the influx is stimulated by hyperpolarization of the cell membrane (Lambert & Hoffmann, 1993). It has, therefore, been suggested that taurine influx via the β-system in the Ehrlich cells is an apparent electroneutral $2Na^+$, $1Cl^-$, 1taurine cotransport, and that it is the negatively charged carrier which makes

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the taurine influx via the β-system sensitive to the membrane potential (Lambert & Hoffmann, 1993).

Cloning of the β-system from human thyroid (Jhiang et al., 1994), Madin Darby Canine Kidney (MDCK) cells (Uchida et al., 1992), rat brain (Smith et al., 1992) and mouse brain (Liu et al., 1992) has revealed that the β-system belongs to a family of very similar Na^+-coupled transport systems each with 12 hydrophobic, transmembrane segments. This family also includes the cotransporters responsible for the reuptake of neurotransmitters, e.g., serotonin, dopamine and γ-aminobutyric acid in the mammalian nervous system (Rudnick & Clark, 1993). According to the definition of consensus phosphorylation sites, established by Kennelly and Krebs (1991), the cloned sequences for the β-system from various cells (Jhiang et al., 1994; Liu et al., 1992; Smith et al., 1992; Uchida et al., 1992) reveal putative phosphorylation sites for a range of kinases including protein kinase C (PKC) and the 3′,5′-cyclic adenosine monophosphate (cAMP) activated protein kinase A (PKA). It has been reported that activation of PKC leads to phosphorylation and inactivation of the β-system in a human choriocarcinoma cell line (JAR cells) (Kulanthaivel et al., 1991) and in human colon cell lines (HT-29 and Caco-2) (Brandsch et al., 1993), whereas an isoproterenol induced increase in cAMP stimulates the taurine influx in rat heart (Huxtable et al., 1980) and addition of 8-bromo-cAMP stimulates the taurine influx in flounder intestine (King et al., 1986).

In this report, we elucidate the role of cAMP, PKA, PKC and phosphatases in the regulation of the initial taurine influx via the β-system in the Ehrlich cells. The cellular cAMP content is varied by manipulation of the adenylate cyclase and the phosphodiesterase activity, and the effect of cAMP on the taurine influx is mimicked by addition of the lipid permeable, nonhydrolyzable cAMP analogue N^6,2′-O-dibutyryl-cAMP (dbcAMP). PKC is activated by addition of phorbol 12-myristate 13-acetate (PMA), whereas phosphatases are inhibited by addition of calyculin A (CLA). A model is proposed, according to which the β-system in the Ehrlich cells exists in three activity states depending on the degree of phosphorylation and on the cellular cAMP level. Parts of this work have been presented as an abstract for the Scandinavian Physiological Society Meeting in Copenhagen in May 1995 (Mollerup & Lambert, 1995).

Materials and Methods

Cell Suspension

Ehrlich ascites tumor cells (hyperdiploid strain), grown for 8 days in the abdominal cavity of female NMRI (Naval Medical Research Institute) mice, were harvested in standard medium containing heparin (2.5 IU × ml^{-1}), washed twice by centrifugation (700 × *g*, 45 sec) in standard medium, and finally resuspended in standard medium (cytocrit 4–6%). Heparin was added in order to avoid clotting of the cells. The cells were preincubated for 30 min before initiation of the experiments. The temperature was kept at 37°C.

Inorganic Non-nutrient Media

Standard medium (in mM): 150 Na^+ and Cl^-; 5.0 K^+; 1.0 Ca^{2+}, Mg^{2+}, SO_4^{2-} and PO_4^{3-}; 3.3 MOPS (3-[N-morpholino]propane sulfonic acid) and TES (N-tris-[hydroxymethyl]methyl-2-aminoethane sulfonic acid) and 5 HEPES (N-[2-hydroxyethyl]-piperazine-N′-[2-ethane] sulfonic acid). Choline medium (in mM): 143 choline; 7.0 K^+; 145 Cl^-; 1 Ca^{2+}, Mg^{2+}, SO_4^{2-} and PO_4^{3-}; 3.3 MOPS and TES; 5 HEPES. KCl medium (in mM): 150 K^+ and Cl^-; 1 Ca^{2+}, Mg^{2+}, SO_4^{2-} and PO_4^{3-}; 3.3 MOPS and TES; 5 HEPES. pH was adjusted to 7.40 in all media at room temperature.

Taurine Influx

The initial taurine influx was estimated as described in details in Lambert and Hoffmann (1993). In brief, ^{14}C-labeled taurine (0.16 μCi × ml^{-1}, 1.5 μM) was added to the cell suspension at time zero and 5 samples (1 ml) of the cell suspension were drawn during the consecutive 5 min for estimation of the ^{14}C-taurine activity in the cells and in the extracellular medium. The cellular ^{14}C-taurine activity was corrected for extracellular trapped activity using ^{3}H-labeled inulin as an marker of the extracellular space (Hoffmann, Simonsen & Sjøholm, 1979). The rate constant for the initial taurine influx (k', $min^{-1} \times g$ medium × *g* cell dry wt^{-1}) was calculated as the slope of a plot of a_c^t/a_m^0 *vs.* time (*see* Fig. 1, upper panel), where a_c^t is the cellular activity of ^{14}C-taurine (cpm × *g* cell dry wt^{-1}) at time *t*, and where a_m^0 is the ^{14}C-taurine activity in the medium (cpm × *g* $medium^{-1}$) at time zero. a_m^0 was estimated by extrapolation of the ^{14}C-taurine activity in the medium during the sampling period. The initial taurine influx (nmol × *g* dry $wt^{-1} \times min^{-1}$) was calculated as the product of k' and the taurine concentration in the medium (μmol × *g* $medium^{-1}$).

Estimation of Cellular cAMP by Reversed Phase High Pressure Liquid Chromatography and by Radioimmunoassay

For estimation of the cellular cAMP content in the Ehrlich cells by reversed phase high pressure liquid chromatography (HPLC) samples (1.5 ml) of the cell suspension (cytocrit 6%) were drawn, centrifuged (15,000 × *g*, 45 sec), and the nucleotides extracted with cold perchloric acid (PCA, final concentration 0.6 mM). After 20 min incubation, and another centrifugation (15,000 × *g*, 10 min), the acidic supernatant, containing the nucleotides, was transferred to new vials and neutralized to pH ≈ 7 by addition of two volumes of freshly prepared tri-N-octylamine (0.5 M) in freon (Khym, 1975). Excess supernatant was removed and the PCA cell pellet was dried for 48 hr (90°C) and weighed. To improve the separation of cAMP from ATP, ADP and AMP these noncyclic nucleotides were precipitated by addition of a small volume (15% v/v) of $ZnSO_4$ and Na_2CO_3 (in a mutual distribution of 1 to 2), whereafter the samples were centrifuged again (Chan & Lin, 1974). Twenty μl of the supernatant was injected into a HPLC system (Pharmacia Biotech) and the nucleotides were separated on a C18-Superpac Sephasil column (4 × 250 mm, 5 μm particles). The mobile phase consisted of 50 mM K_2HPO_4, pH 5.5 and 10% methanol (*v/v*), the flow velocity was 0.8 ml/min and the compounds were detected at 260 nm. The absolute cAMP content was estimated from chromatograms of cAMP standards in the range 0.25 μM to 8.0 μM.

The cAMP standard curve was linear in the whole range examined, and the recovery of cAMP, estimated by addition of cAMP as an internal standard before neutralization and precipitation of unwanted nucleotides, was ≈100%. The cellular cAMP content (pmol × mg PCA dry wt^{-1}) was converted to the protein content (pmol × mg $protein^{-1}$) by division with 0.81 (mg protein × mg PCA dry wt^{-1}). This factor (0.81 ± 0.03) was estimated in 4 separate experiments, where cellular protein was measured by the method of Lowry et al. (1951), using bovine serum albumin as a standard, in parallel with the estimation of the PCA dry weight.

In experiments where the cellular cAMP content (pmol × mg $protein^{-1}$) changed rapidly with time we estimated the cAMP content by radioimmunoassay (RIA) using a cAMP RIA-kit (dual range) from Amersham International. An aliquot of the cell suspension (0.4 ml, cytocrit 5.0%) was drawn and cold PCA was added to a final concentration of 0.3 M. The acid was extracted using tri-N-octylamine in freon (*see above*). For estimation of the extracellular cAMP content (pmol × ml $medium^{-1}$) a sample (0.4 ml) was drawn, centrifuged (15,000 × *g*, 45 sec) and the supernatant (cell free medium) handled as the samples for the estimation of the cellular cAMP content.

Estimation of the Membrane Potential

The membrane potential in the Ehrlich cells was estimated from the fluorescence intensity of the dye 1,1-dipropyloxa dicarbocyanine iodide ($DiOC_3$-(5)) by the method described in Lambert et al. (1989). In brief, the dye was added to the cell suspension (cytocrit 0.25%) at a final concentration of 1.6 μM. Aliquots of the suspension were transferred to a cuvette with a teflon-coated magnet stirrer, and placed in the thermostatically controlled (37°C) cuvettehouse of a luminescence spectrometer (Perkin Elmer, LS-5). Excitation and emission wavelengths were 577 nm and 605 nm, respectively, and the slit widths were 5 nm. For calibration of the fluorescence signal, we suspended cells in Na^+-free, K^+/choline media, where the extracellular K^+ concentration was varied isosmotically, and measured the fluorescence in the presence of the cation ionophore gramicidin. The calibration curve was achieved as a plot of the fluorescence measured in the presence of gramicidin *vs.* the Nernst equilibrium potential for K^+, estimated from measured intracellular and extracellular K^+ concentrations.

Chemicals

Stock solutions were prepared as follows (in mM): Calyculin A (20 μM) (Alamone Labs LTD, Jerusalem, Israel); H-89 (5) and H-7 (5) (Biomol Res. Lab., Plymouth Meeting, PA); gramicidin (1), valinomycin (1.2), forskolin (50), and phorbol 12-myristate 13-acetate (20 μM) (Sigma Chemicals, St. Louis, MO) were all dissolved in 96% ethanol and stored at –22°C. Theophylline (40) and N^6,2′-O-dibutyryl 3′,5′-cyclic adenosine monophosphate (100) (Sigma Chemicals) were dissolved in standard medium and kept at –22°C. Tri-N-octylamine (Sigma Chemicals) was stored at room temperature and mixed with freon (1,1,2-trichlorotrifluoro ethane) (Aldrich-Chemie, Steinheim, Germany) just before use. Stock solutions of 3′,5′-cyclic adenosine monophosphate (4) (Sigma Chemicals) were prepared in Millipore-Q-plus water (HPLC-grade) in small portions and stored at –22°C. Bovine serum albumin (fraction V) (Sigma Chemicals) was dissolved in standard medium and kept at 2–5°C. ^{3}H-labeled inulin (dissolved in 1 mM TES) and ^{14}C-labeled taurine (DuPont NEN, Kastrup, Denmark) were kept at –22°C and diluted in standard medium before use. 1,1-dipropyloxa dicarbocyanine iodide (1.2) (Molecular Probes, Junction City, OR) was dissolved in 96% ethanol and kept at –22°C. Licrosolv methanol (HPLC-grade) was obtained from Merck (Darmstadt, Germany).

Statistics

The results are given as mean ± SEM with the number of experiments (*n*) indicated. For statistical evaluation we used the Student's *t*-test or the paired *t*-test and a significance level of 0.05.

Results

Activation of PKC Reduces the Activity of the β-System

It has previously been shown that pretreatment of JAR cells, HT-29 cells and Caco-2 cells with phorbol ester (PMA) decreases the taurine influx, and that staurosporine, a potent kinase inhibitor, abolishes the effect of PMA (Kulanthaivel et al., 1991; Brandsch et al., 1993). Since phorbol esters are known to activate most isoforms of PKC (Nishizuka, 1992) this is taken to indicate that the activity of the β-system is diminished following PKC mediated phosphorylation. To assess whether activation of PKC also reduced the activity of the β-system in the Ehrlich cells, we preincubated the Ehrlich cells for 10 min with PMA prior to the initiation of the taurine influx experiment. Figure 1 (upper panel) demonstrates that the initial taurine influx is significantly reduced by 40 nM PMA. From the slopes of the influx curves it is estimated that the initial taurine influx in Ehrlich cells suspended in standard medium with 1.5 μM taurine is reduced to approximately 70% of the original value following preincubation with 200 nM PMA (Fig. 1, lower panel). However, the inhibitory effect of PMA on the initial taurine influx is significantly impaired in the presence of 30 μM H-7, which is reported to inhibit PKC as well as PKA (Hidaka & Kobayashi, 1993) (Fig. 1, lower panel). Since taurine influx at low extracellular taurine concentrations (1.5 μM) is solely via the β-system (Lambert, 1984) and since H-7 has no effect on the initial taurine influx in the absence of PMA (*see* Fig. 5, upper panel) it is suggested that phosphorylation by PKC reduces the activity of the β-system in the Ehrlich cells.

Accumulation of cAMP Increases the Activity of the β-System

To investigate whether the cAMP-sensitive PKA was involved in the regulation of the β-system in the Ehrlich cells, we estimated the cAMP content and the initial taurine influx after addition of forskolin, theophylline or theophylline plus forskolin. Forskolin is known to stimulate the mammalian adenylate cyclases, independent of G-protein activation, whereas theophylline is an unselec-

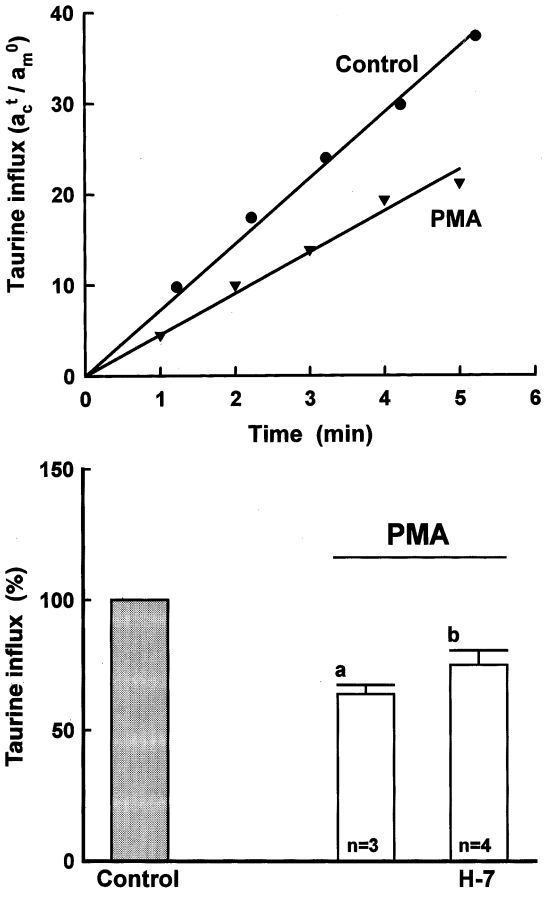

Fig. 1. Effect of phorbol 12-myristate 13-acetate (PMA) and the kinase inhibitor H-7 on the initial taurine influx in Ehrlich cells. *Upper panel:* ^{14}C-labeled taurine (0.16 μCi × ml^{-1}, 1.5 μM) was added to the cell suspension (cytocrit 4–6%) at time zero, and the increase in the cellular ^{14}C-taurine activity was followed with time in control cells (●) and in cells treated with PMA (40 nM, 10 min, ▼). a_c^t is the cellular ^{14}C-taurine activity at time t (cpm × *g* cell dry wt^{-1}) and a_m^0 is the ^{14}C-taurine activity in the medium at time zero (cpm × *g* medium^{-1}). The figure is representative of four separate experiments. *Lower panel:* The increase in cellular ^{14}C-taurine activity was followed with time in cells treated with PMA (200 nM, 10 min) in the absence and in the presence of H-7 (30 μM, 8 min). The initial taurine influx (nmol × *g* dry wt^{-1} × min^{-1}) was estimated as the product of the taurine concentration in the medium (μmol × *g* medium^{-1}) and the rate constant for the initial taurine influx (k′, min^{-1} × *g* medium × *g* cell dry wt^{-1}), i.e., the slope of the plot of a_c^t/a_m^0 *vs.* the time. Flux values are given in percent of the initial influx in control cells ± SEM. [a]PMA significantly reduces the initial taurine influx ($P < 0.007$, paired *t*-test). [b]The effect of PMA on the initial taurine influx is significantly reduced in the presence of H-7 ($P < 0.03$, Student's *t*-test).

tive phosphodiesterase inhibitor (Thompson, 1991). In Fig. 2 it is seen that the cellular cAMP content increases within the first minute following addition of forskolin, whereafter the cAMP content reaches a plateau. The effect of forskolin on the cAMP content is enhanced if the Ehrlich cells are preincubated with theophylline (Fig. 2). The cellular cAMP content in unpertubated Ehrlich cells

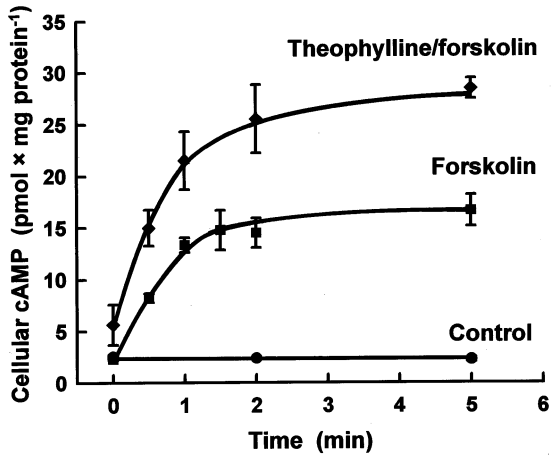

Fig. 2. Effect of forskolin on the cellular cAMP content in Ehrlich cells. Forskolin (10 μM) was added to cells preincubated for 30 min in the absence (■) or in the presence of theophylline (0.5 mM, ◆). Control cells (●) were not exposed to forskolin or theophylline. Cellular cAMP was estimated by RIA. Values are given as mean values ± SEM of three independent sets of experiments. The curves are drawn by hand. The cellular cAMP content in forskolin treated cells are at all time points larger in presence of theophylline ($P < 0.02$, Student's *t*-test).

is estimated at 2.4 ± 0.2 pmol × mg protein^{-1} by the RIA technique and at 1.0 ± 0.3 pmol × mg protein^{-1} by the HPLC technique (Table), which is in the same range as the 0.6 pmol × mg protein^{-1} previously reported by Chayoth (1986). Addition of theophylline (0.5 mM) and forskolin (10 μM) increases the cellular cAMP content to about 6 pmol × mg protein^{-1} and to 13–17 pmol × mg protein^{-1}, respectively (Table). The effects of forskolin and theophylline on the cAMP content indicate, as expected for a mammalian cell, that the cAMP level in the Ehrlich cells is regulated by adenylate cyclase and by phosphodiesterase activity. From Fig. 3 it is seen, that addition of forskolin (10 μM) stimulates the initial taurine influx in the Ehrlich cells, and that dbcAMP (0.2 mM) mimics the effect of forskolin. From inspection of the Table, it is seen that there is a positive relationship between the cAMP content of the Ehrlich cells and the initial taurine influx. It is estimated that an increase in the cAMP content from 2.4 to 28 pmol × mg protein^{-1} increases the initial taurine influx by almost 50% (control cells compared to theophylline plus forskolin treated cells, Table). Thus, an increased level of cAMP stimulates the initial taurine influx in Ehrlich cells. It is noted that preincubation of the Ehrlich cells with 0.5 mM db-cAMP results in a 63% increase in the initial taurine influx (Table). However, if the concentration of forskolin is increased from 10 μM to 30 μM the taurine influx is not increased further, it even seems to be slightly reduced (*data not shown*), indicating that 30 μM forskolin could have a toxic effect in Ehrlich cells.

It has been demonstrated that taurine influx via the β-system in Ehrlich cells is totally Na^+-dependent and

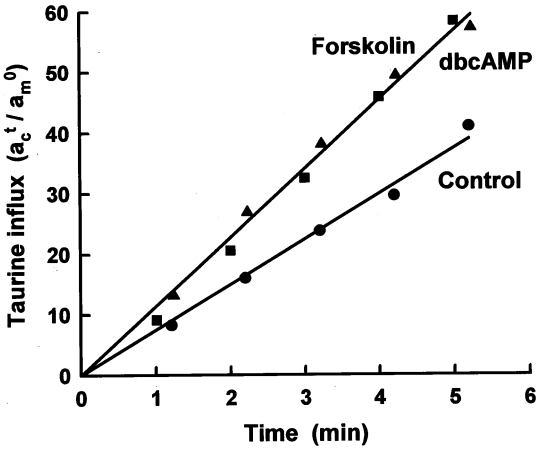

Fig. 3. Stimulation of the initial taurine influx in Ehrlich cells with forskolin and dbcAMP. ^{14}C-labeled taurine (0.16 μCi × ml^{-1}, 1.5 μM) was at time zero added to the cell suspension (cytocrit 4–6%), and the increase in the cellular ^{14}C-taurine activity was followed with time in control cells (●), and in cells treated with forskolin (10 μM, 1 min, ■) or dbcAMP (0.2 mM, 1 min, ▲). For details, *see* Fig. 1 legend. The figure is representative of five separate experiments.

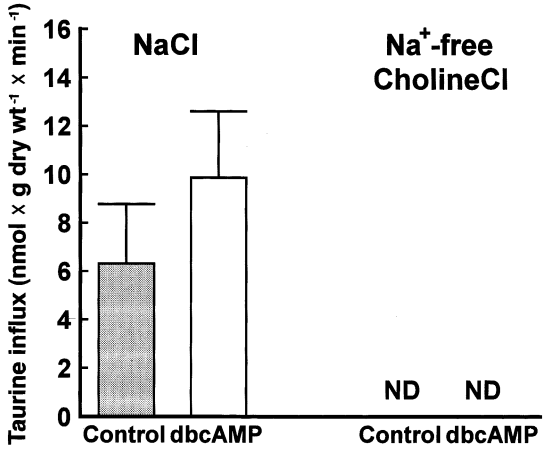

Fig. 4. The effect of dbcAMP on the initial taurine influx in Ehrlich cells suspended in NaCl medium and in *nominally* Na^+-free cholineCl medium. Cells, preincubated in standard medium (cytocrit 4–6%) for 30 min, were centrifuged (700 × *g*, 45 sec), washed and resuspended in either standard NaCl medium or in cholineCl medium. The cells were then incubated for another 5 min in the absence or in the presence of dbcAMP (0.5 mM). The initial taurine influx was estimated as described in the Fig. 1 legend and given as the mean ± SEM of four (NaCl medium) or three (cholineCl medium) sets of experiments. ND indicates that no taurine influx was detected.

that the initial taurine influx is zero in the absence of extracellular Na^+ (Lambert, 1984). This is confirmed in Fig. 4, which shows the initial taurine influx in Ehrlich cells suspended in standard NaCl medium and in nominantly Na^+-free cholineC1 medium. However, the Ehrlich cells also possess two apparently Na^+-independent systems, i.e., a taurine efflux system, which resembles the β-system with respect to potential- and pH-sensitivity (Lambert & Hoffmann, 1993) and the taurine channel, which is activated by cell swelling (Lambert & Hoffmann, 1994). To see whether the cAMP-induced taurine influx was Na^+-dependent, we estimated the effect of dbcAMP on the initial taurine influx in the presence and in the absence of Na^+ in the extracellular medium. Figure 4 demonstrates that dbcAMP does not provoke any detectable taurine influx in the absence of Na^+, indicating that the cAMP-induced taurine influx is Na^+-dependent, i.e., cAMP stimulates the β-system in the Ehrlich cells.

Effect of PKA Inhibitors on the cAMP-induced Activation of the β-System

To further investigate whether the activation of the β-system by cAMP could be due to phosphorylation by

Table. Effect of theophylline, forskolin and dbcAMP on the cellular cAMP level and the initial taurine influx in Ehrlich cells

	cAMP plateau level (pmol × mg protein^{-1})		Taurine influx (%)
	RIA	HPLC	
Control	2.4 ± 0.2 (9)	1.0 ± 0.3 (12)	100
Theophylline	5.7 ± 1.9 (3)[a]		118 ± 22 (2)
Forskolin	17 ± 2 (3)[b]	13 ± 2 (4)[d]	133 ± 16 (16)[e]
Theophylline/forskolin	28 ± 1(3)[c]		149 ± 14 (3)[f]
dbcAMP			163 ± 22 (4)[g]

Cells were preincubated with theophylline (0.5 mM, 30 min), forskolin (10 μM, 1 min), theophylline plus forskolin, or dbcAMP (0.5 mM, 5 min). The cellular cAMP content was estimated by RIA and by the HPLC technique (*see* Materials and Methods). The initial taurine influx was estimated as described in the Fig. 1 legend and given in percent of the initial influx in unpertubated cells. All values are given as means ± SEM with the number of experiments indicated in the parentheses. [a]Significantly larger than the control value ($P < 0.001$, Students *t*-test). [b]Significantly larger than the value for theophylline-treated cells ($P < 0.01$, Student's *t*-test). [c]Significantly larger than the value for forskolin-treated cells ($P < 0.001$, Student's *t*-test). [d]Not different from the value estimated by RIA ($P > 0.1$, Student's *t* test). [efg]Significantly larger than the control value ($P < 0.04$, Student's *t*-test).

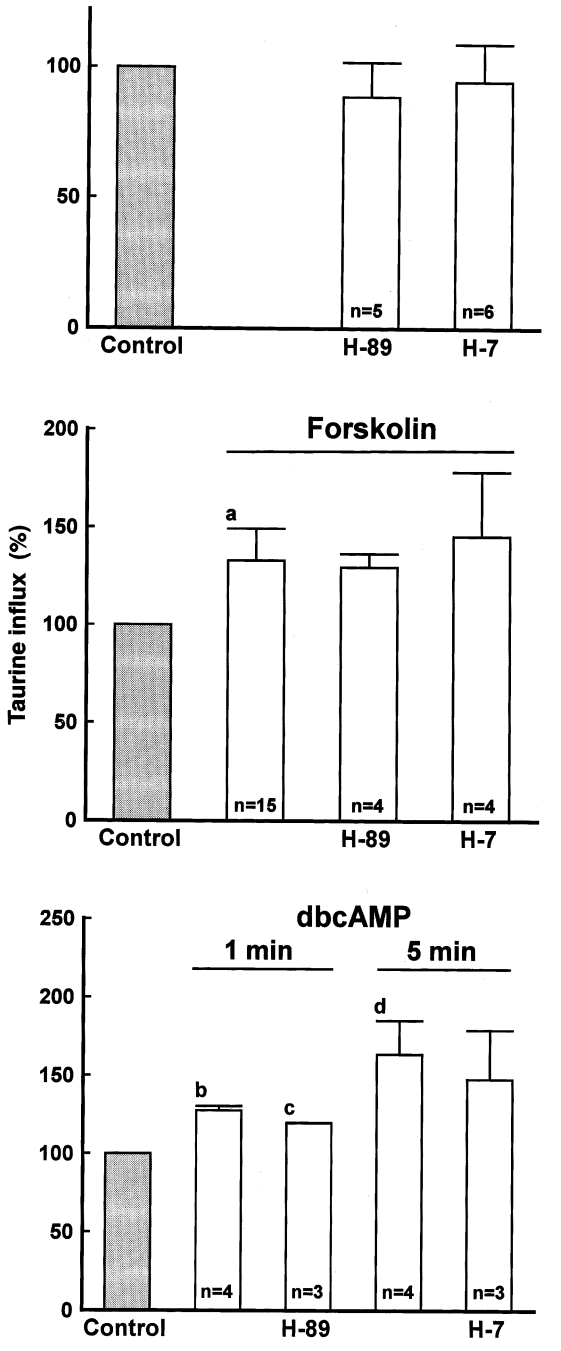

Fig. 5. The effect of H-89 and H-7 on the forskolin- and the dbcAMP-induced taurine influx in Ehrlich cells. Cells were preincubated in the absence and in the presence of H-89 (10 μM, 12 min) or H-7 (30 μM, 8 min), and the initial taurine influx was estimated as described in the legend to Fig. 1. *Upper panel:* The influx was estimated in cells treated with H-89 or H-7. *Middle panel:* The influx was estimated in cells stimulated with forskolin (10 μM, 1 min) in the absence and in the presence of H-89 or H-7. *Lower panel:* The influx was estimated in cells simulated with dbcAMP (0.5 mM, 1 or 5 min) in the absence and in the presence of H-89 or H-7. The initial taurine influx is in all cases given in percent of the initial taurine influx in unpertubated control cells ± SEM. [a]Forskolin significantly increases the initial taurine influx ($P < 0.001$, paired *t*-test). [bd]dbcAMP significantly increases the initial taurine influx ($P < 0.01$, paired *t*-test). [c]H-89 significantly decreases the effect of dbcAMP on the initial taurine influx ($P < 0.005$, Student's *t*-test).

the cAMP activated PKA, we studied the effect of the PKA inhibitors H-89 and H-7 on the forskolin- and the dbcAMP-induced taurine influx. Preincubation with H-89 or H-7 does not affect the initial taurine influx in the absence (Fig. 5, upper panel) or in the presence of forskolin (Fig. 5, middle panel). On the other hand, the stimulation of the initial taurine influx by dbcAMP is inhibited significantly by H-89 but not significantly by H-7 (Fig. 5, lower panel). The data with forskolin, H-89 and H-7 indicate that the cAMP-induced activation of the β-system could be direct, i.e., independent of a PKA-mediated phosphorylation, whereas the data with dbcAMP and H-89 indicate that the cAMP-induced activation of the β-system could involve activation of PKA. At present, the cAMP concentration needed for activation of PKA in Ehrlich cells is unknown, it is therefore not possible to establish if the larger activity of the β-system seen in dbcAMP treated cells compared to forskolin treated cells (*see* Table) as well as the lack of effect of H-89 and H-7 in forskolin treated cells (Fig. 5, middle panel), reflect that the concentration of cAMP in forskolin treated cells is too low to activate a H-89- and H-7-sensitive PKA.

The Effect of PMA and dbcAMP on the Taurine Influx is not Secondary to Membrane Potential Changes

Taurine influx via the β-system in Ehrlich cells is stimulated by hyperpolarization of the cell membrane (Lambert & Hoffmann, 1993). If the inhibition of the taurine influx induced by PMA (Fig. 1) and the activation of the taurine influx induced by cAMP (Fig. 3) are secondary to changes in the cell membrane potential one would expect the cell membrane to depolarize following addition of PMA and to hyperpolarize following addition of dbcAMP. The resting cell membrane potential was estimated at −63 ± 4 mV ($n = 15$) in unpertubated control cells, which corresponds to the −61 mV published by Lambert and coworkers (1989). However, from Fig. 6 it appears that addition of forskolin to the cells does not change the membrane potential (upper panel), whereas addition of dbcAMP to the cells depolarizes the cell membrane slightly (depolarization less than 5 mV, lower panel). Thus, the effect of dbcAMP and forskolin on the taurine influx (Figs. 3, 4, 5 and Table) cannot be explained by membrane potential changes. On the other hand, incubation of the Ehrlich cells for 10–30 min with 200 nM PMA depolarizes the cells to −60 ± 2 mV ($n = 4$). A depolarization of 3 mV should reduce the taurine in-

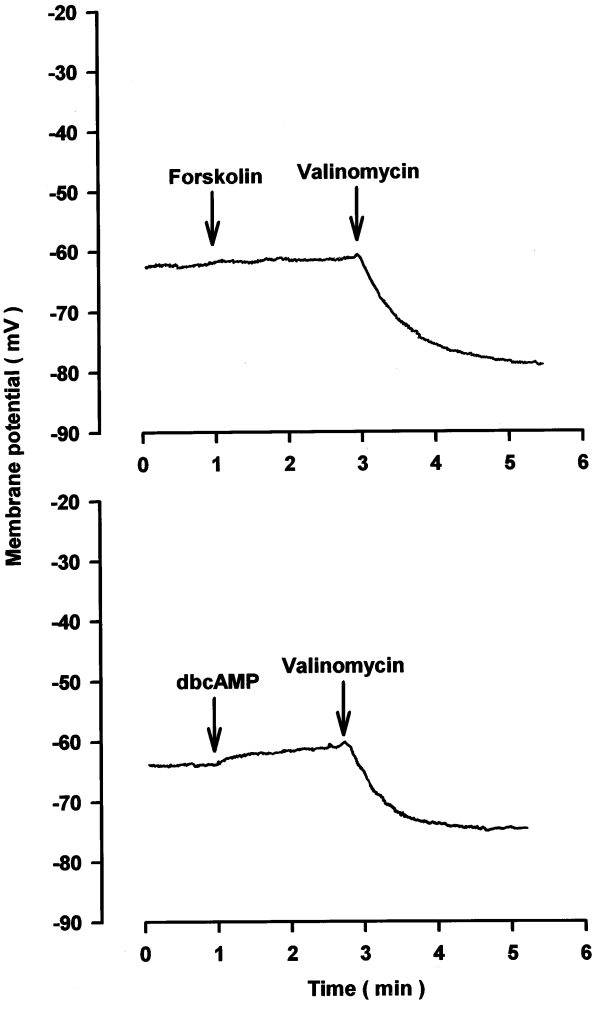

Fig. 6. The effect of forskolin and dbcAMP on the membrane potential in Ehrlich cells. The fluorescent dye $DiOC_3$-(5) (1.6 μM) was added to cells suspended in standard medium (cytocrit 0.25%) and the fluorescence was followed with time. Absolute membrane potentials were obtained by calibration of the fluorescence signal in a Na^+-free K^+/choline$^+$ media (*see* Materials and Methods). *Upper panel:* Forskolin (10 μM) was added as indicated by the arrow. *Lower panel:* dbcAMP (0.5 mM) was added as indicated by the arrow. Valinomycin (1.2 μM) was added at the end of each experiment in order to verify that the potassium gradient was intact—as evidenced by the strong hyperpolarization of the cell membrane. The curves are representative of three independent experiments.

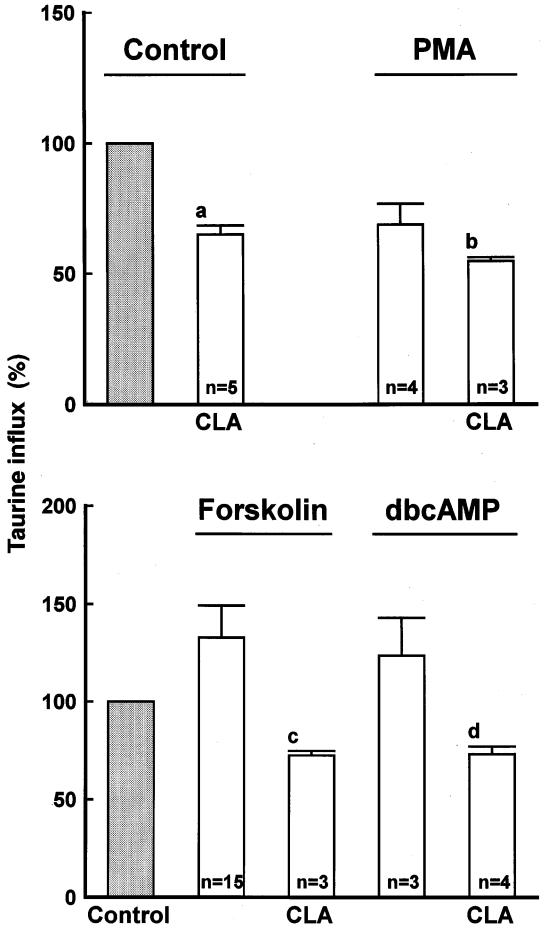

Fig. 7. The effect of the phosphatase inhibitor CLA on the initial taurine influx in Ehrlich cells. Cells were prepared and the initial taurine influx estimated as described in the Fig. 1 legend. *Upper panel:* The effect of CLA (100 nM, 1.5 min) on the influx was estimated in the absence or in the presence of PMA (40 nM, 10 min). *Lower panel:* The effect of CLA (100 nM, 1.5 min) on the influx was estimated in the absence or in the presence of forskolin (10 μM, 1 min) or dbcAMP (0.2 mM, 1 min). The initial taurine influx is in all cases given in percent of the initial taurine influx in unpertubated control cells ± SEM. [a]CLA significantly decreases the taurine influx ($P < 0.006$, Paired *t*-test). [b]PMA significantly potentiates the effect of CLA ($P < 0.03$, Student's *t*-test). [cd]CLA significantly attenuates the effect of dbcAMP or forskolin on the initial taurine influx ($P < 0.02$, Student's *t*-test).

flux by approximately 10% (*see* Fig. 5 in Lambert and Hoffmann, 1993). Thus, the 30% inhibition of the taurine influx seen in Fig. 1, which is induced by a lower PMA concentration (40 nM PMA, Fig. 1), is not solely secondary to a depolarization of the cell membrane.

Role of Phosphatases in Regulation of the Activity of the β-System

It has recently been demonstrated that treatment of Ehrlich cells with 100 nM CLA leads to activation of an otherwise quiescent Na^+,K^+,$2Cl^-$ cotransporter (Jacobsen, Jensen & Hoffmann, 1994). CLA is a potent inhibitor of protein phosphatase 1, 2A and 3 (PP1, PP2A and PP3) (Honkanen et al., 1994). To see whether the β-system was regulated by phosphatases, we exposed the Ehrlich cells to CLA. From Fig. 7 (upper panel) it is seen, that addition of CLA (100 nM) results in a 30% inhibition of the initial taurine influx, indicating that dephosphorylation by a CLA-sensitive phosphatase stimulates the activity of the β-system. Furthermore, the initial taurine influx is inhibited to a larger extent when the Ehrlich cells are exposed to CLA plus PMA (Fig. 7, upper

panel). These results indicate, that treatment of the Ehrlich cells with CLA and PMA, i.e., inhibition of a CLA-sensitive phosphatase and stimulation of PKC in both cases lead to inactivation of the β-system. Forskolin and dbcAMP stimulate the taurine influx in CLA-treated cells, however, the resulting initial taurine influx is about 30% lower than the influx estimated in unpertubated control cells (Fig. 7, lower panel). Thus, inactivation of CLA-sensitive phosphatases prevent maximal activation of the β-system by forskolin and by dbcAMP, i.e., phosphatases are important in the regulation of the activity of the β-system in the Ehrlich cells.

Discussion

The β-System is Stimulated by cAMP

In the past, a regulative role for cAMP and the cAMP activated PKA has been ascribed to a number of different membrane proteins, including K^+-channels (Scornik et al., 1993), Cl^--channels (Levitan, 1994), Ca^{2+}-channels (Perezreyes et al., 1994), the Na^+, K^+, $2Cl^-$-cotransporter (Palfrey & Pewitt, 1993), as well as the Na^+, K^+ ATP-ase (Bertorello & Katz, 1993). In addition, a role for PKA mediated phosphorylation in the regulation of neurotransmitter cotransporters has been suggested (Cool et al., 1991), and this is of specific interest because these transporters have significant structural and topographical similarity with the taurine transporting β-system (Shafqat et al., 1993).

The data presented in the Table and Figs 2, 3 and 5 indicate that it is possible to clamp the cellular cAMP content in Ehrlich cells by use of forskolin and theophylline, and that an increase in the cellular cAMP content is accompanied by an increase in the initial taurine influx. The effect of cAMP on the initial taurine influx is mimicked by dbcAMP, and since the effect of dbcAMP on the taurine influx is totally Na^+-dependent (Fig. 4), it is suggested that dbcAMP and cAMP stimulate the Na^+-dependent β-system in the Ehrlich cells. It has previously been demonstrated that a reduction in the Na^+ conductance, an increase in the K^+ conductance or stimulation of Na^+, K^+ ATP-ase in the Ehrlich cells hyperpolarize the cell membrane (*see* Lambert et al., 1989), and that hyperpolarization of the cell membrane stimulates the initial taurine influx in the Ehrlich cells (Lambert & Hoffmann, 1993). However, the cell membrane is not hyperpolarized following addition of forskolin and dbcAMP (Fig. 6), indicating that the effect of cAMP on taurine influx via the β-system is not secondary to a cAMP-induced change in cation conductances or ATP-ase activity, i.e., cation gradients. This is in accordance with previous studies by Azari and Huxtable (1980), in which it was demonstrated that the cAMP-induced taurine transport in the rat heart is not secondary to changes in cation movements.

To envisage the involvement of PKA mediated phosphorylation, in the stimulatory effect of cAMP on the β-system, we used the kinase inhibitors H-89 and H-7 in combination with forskolin and dbcAMP. The resulting data are not unequivocal, i.e., the effect of forskolin on the initial taurine influx is apparently insensitive to H-89 and H-7, whereas the effect of dbcAMP is partly inhibitable by the kinase inhibitors (Fig. 5). Thus, assuming that the kinase inhibitors are functional in the Ehrlich cells, cAMP seems both to stimulate the β-system directly, i.e., without involvement of protein kinases, and indirectly, i.e., via PKA-mediated phosphorylation. It is noted that only a short preincubation period is needed for stimulation of the initial taurine influx in the Ehrlich cells by forskolin or dbcAMP, indicating that *de novo* protein synthesis is most probably not involved in the process. In this case, the observation that prolonged incubation with dbcAMP increases the effect of dbcAMP on taurine influx (compare influx after 1 and 5 min incubation with dbcAMP in Fig. 5, lower panel) seems to reflect a low permeability of the Ehrlich cells to dbcAMP. Interestingly, cAMP stimulated reorganization of F-actin has been described as a prerequisite for insertion of Cl^--channels and Cl^- secretion in T84 colon carcinoma cells (Shapiro et al., 1991). At the moment we are investigating whether cytochalasin B, which disrupts microfilaments, affects the cAMP-induced stimulation of the β-system.

The β-System is Inhibited after Activation of PKC

A role for PKC in the regulation of the β-system from different human cell lines has been given (Kulanthaivel et al., 1991; Brandsch et al., 1993). Brandsch and coworkers (1993) found that preincubation of HT-29 cells with PMA decreased their capacity for taurine transport as well as the affinity of the carrier to taurine, and they suggested that PKC phosphorylated and inactivated the taurine transporter. The initial taurine influx in Ehrlich cells is also reduced after activation of PKC (Fig. 1). Furthermore, this inhibition of the initial taurine influx induced by PMA is impaired in the presence of the kinase inhibitor H-7 (Fig. 1). Our data together with data from other cell lines (*see* Introduction), therefore, lead us to suggest, that the β-system in the Ehrlich cells can be phosphorylated on one or more of its putative PKC phosphorylation sites, and that phosphorylation by PKC inactivates the transport system. At present we are investigating whether the PKC-induced reduction in transport activity is due to a modification of the membrane potential-sensitivity of the empty carrier (*see* Introduction), a reduced transport capacity, or a reduced affinity of the carrier for taurine, Na^+ or Cl^-.

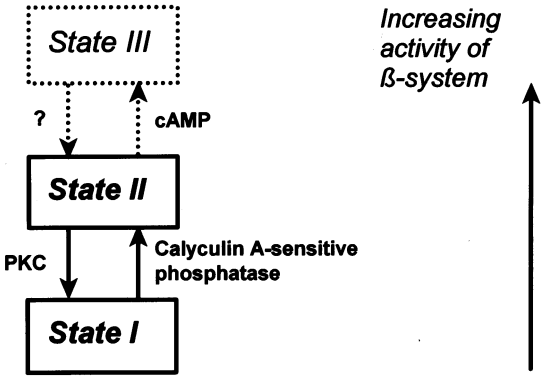

Fig. 8. Model for the β-system in the Ehrlich cells. *See* text for details.

The β-System is Regulated by a CLA-sensitive Phosphatase

The β-system in Ehrlich cells is inactivated after addition of the phosphatase inhibitor CLA (Fig. 7). Since CLA does not affect the membrane potential of the Ehrlich cells (*data not shown*) the inhibition of the β-system by CLA is not secondary to a depolarization of the cell membrane. It is, therefore, suggested that the activity of the β-system in unpertubated Ehrlich cells is partly maintained by a CLA-sensitive phosphatase. Furthermore, treating the Ehrlich cells with CLA in the presence of PMA decreases the activity of the β-system to an even larger extent (Fig. 7). Since the CLA-sensitive phosphatases (PP1 and PP2A) are often the enzymes that reverse the actions of PKC (Cohen, Holmes & Tsukitani, 1990) we take the apparent synergy between the effect of PKC activation and inhibition of CLA-sensitive phosphatases to indicate, that the effect of the CLA-sensitive phosphatases and PKC is on the same site of phosphorylation on the β-system or on a regulator of the β-system. This leads us to propose a model for the β-system in the Ehrlich cells (*see* Fig. 8) according to which the system exists in three states of activity: *State I*, a phosphorylated state with low transport activity, *State II*, a dephosphorylated state with normal transport activity, and *State III*, a state with high transport activity seen after stimulation with cAMP-mobilizing agents and dbcAMP. Transition from *State II* to *State I* is mediated by PKC and transition from *State I* to *State II* is mediated by a CLA-sensitive phosphatase (probably PP1, PP2A or PP3). Since the CLA mediated inactivation of the β-system prevents maximal activation by cAMP (Fig. 7) it appears that transition to *State III* preferentially occurs from *State II*.

Phosphorylation of the β-System in Ehrlich Cells—Physiological Relevance

It is of great importance to most mammalian cells to regulate their cell volume and to avoid pathological states induced by cell swelling or cell shrinkage (Hoffmann, Simonsen & Lambert, 1993). Ehrlich cells swell when exposed to a hypotonic medium, and within minutes they regain their normal cell volume by a net loss of K^+, Cl^- and organic osmolytes (taurine), followed by cell water (*see* Hoffmann et al., 1993). Since the medium taurine concentration increases from 0.07 mM to 1.04 mM after exposure of Ehrlich cells to a hypotonic medium with half of the original osmolarity (Hoffmann & Lambert, 1983) one would expect, due to this increased substrate availability, that the taurine influx via the β-system should increase about 40–50 times (*see* Lambert, 1984). However, the taurine influx via the β-system is significantly reduced during the volume regulatory response (Hoffmann & Lambert, 1983) most likely due to the reduced extracellular Na^+ concentration. Furthermore, reducing the osmolality of the medium, without a concomitant reduction of the Na^+ concentration, reduces in it self the taurine influx (Hoffmann & Lambert, 1983). According to Minton (1994), an increase in cell volume is associated with a corresponding decrease in the concentration of all impermeant intracellular species, one of which could regulate a cellular enzyme or a membrane transporter. Recently, Larsen and coworkers (1994) demonstrated that PKC is slightly activated in Ehrlich cells after hypotonic cell swelling. We, therefore, propose that a reduced phosphatase activity and an increased PKC activity could play an important physiological role in the swelling-induced inactivation of the β-system whereby the Ehrlich cells avoid (re)uptake of taurine during the volume regulatory response.

This work has been supported by the Danish Natural Research Council and by the NOVO foundation. Steinar M. Thoroed is acknowledged for critical reading of the manuscript, and Karen Dissing is acknowledged for her technical assistance.

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