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ORIGINAL ARTICLE

Regulation of taurine homeostasis by protein kinase CK2 in mouse fibroblasts

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Abstract Increased expression of the ubiquitous serine/threonine protein kinase CK2 has been associated with increased proliferative capacity and increased resistance towards apoptosis. Taurine is the primary organic osmolyte involved in cell volume control in mammalian cells, and shift in cell volume is a critical step in cell proliferation, differentiation and induction of apoptosis. In the present study, we use mouse NIH3T3 fibroblasts and Ehrlich Lettré ascites tumour cells with different CK2 expression levels. Taurine uptake via the Na^+ dependent transporter TauT and taurine release are increased and reduced, respectively, following pharmacological CK2 inhibition. The effect of CK2 inhibition on TauT involves modulation of transport kinetics, whereas the effect on the taurine release pathway involves reduction in the open-probability of the efflux pathway. Stimulation of PLA_2 activity, exposure to exogenous reactive oxygen species as well as inhibition of protein tyrosine phosphotases (PTP) potentiate the swelling-induced taurine loss. Inhibition of PI3K and PTEN reduces and potentiates swelling-induced taurine release, respectively. Inhibition of CK2 has no effect on PLA_2 activity and ROS production by NADPH oxidase, whereas it lifts the effect of PTEN and PTP inhibition. It is suggested that CK2 regulates the taurine release downstream to known swelling-induced signal transducers including PLA_2, NADPH oxidase and PI3K.

Keywords Regulatory volume decrease · TBCA · DMAT · SLC6A6 · Volume sensitive organic osmolyte channel

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Introduction

Cell volume control

Correct cell volume control is essential for normal cell function and cell fate, including proliferation and apoptosis (Hoffmann et al. 2009; Lambert et al. 2008; Poulsen et al. 2010). Animal cells are highly permeable to water due to the presence of aquaporins, and the cell volume is controlled by the cellular contents of osmotically active compounds and by the osmolarity of the extracellular compartment. As a consequence, shift in the intracellular or the extracellular osmolarity elicits an instant change in cell volume which is dictated and limited by the water permeability. Most mammalian cells activate volume restoring processes concomitantly to change in cell volume, i.e. swollen and shrunken cells regain their original volume by the volume regulating processes, designated regulatory volume decrease (RVD) and regulatory volume increase (RVI), respectively. RVD involves activation of KCl cotransporters, K^+ and Cl^- channels together with transporters for organic osmolytes. RVI, on the other hand, involves activation of Na-K-2Cl cotransport, Na/H exchange, nonselective cation channels, and Na-dependent transporters for organic osmolytes. For a detailed review of osmo-sensing and characterization of the volume regulatory machinery in mammalian cells under

physiological and patophysiological conditions, see (Hoffmann et al. 2009; Lambert et al. 2008; Lang 2007).

Taurine homeostasis

Taurine is an inert β-amino acid (amino ethane sulphonic acid) that contributes significantly to the cellular pool of organic osmolytes in mammalian cells; hence net uptake or net loss of taurine is an important element in cell volume control. It has been estimated that normal intracellular taurine concentration is in the range of 5–50 mM and that taurine in some tissues such as heart and retina constitutes over 50% of the free amino acid pool (Lourenço and Camilo 2002). Mammalian cells accumulate taurine from the extracellular compartment via the β-amino acid transporter TauT (SLC6A6) and release taurine via an as yet unidentified, volume-sensitive organic osmolyte channel. Taurine uptake via TauT is strictly Na^+ dependent, i.e. no taurine is taken up in the absence of extracellular Na^+. In a recent paper it was demonstrated that pharmacological inhibition of CK2 in normal NIH3T3 fibroblasts increased TauT′s affinity towards Na^+ and reduced the Na^+: taurine stoichiometry from 2.6 to 1.9 which resulted in an increased maximal rate for taurine uptake (Jacobsen et al. 2008). It was proposed that the constitutively active CK2 limits the taurine uptake in mammalian cells through phosphorylation of TauT or a putative regulator of TauT (Jacobsen et al. 2008).

The volume-sensitive taurine release system is activated within minutes after exposure to hypoosmotic medium and the concomitant taurine transport is best characterized as a Na^+ independent, non-saturable, passive transport process which is sensitive to the anion channel blockers 4,4′-Diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) and tamoxifen (Lambert 2004; Wang et al. 2004). Activation of the volume-sensitive taurine release in, e.g. NIH3T3 fibroblasts, Ehrlich ascites tumour cells (EAT) and Ehrlich Lettré ascites tumour (ELA) cells following osmotic cell swelling involves an unknown sensor of cell volume and activation of phospholipase A_2 (PLA_2) subtypes (Hoffmann et al. 2009; Lambert and Pedersen 2006; Lambert et al. 2006; Lambert and Hoffmann 1993; Lambert 2007). The subsequent intracellular signalling involves arachidonic acid mobilization and most likely generation of lysophospholipids, oxidation of the unsaturated fatty acid via a 5-lipoxygenase (5-LO) to eicosanoides, as well as generation of reactive oxygene species (ROS). Arachidonic acid is released from the nuclear membrane in the NIH3T3 fibroblasts (Pedersen et al. 2006), and it has been demonstrated that lysophosphatidic acid, when added in nanomolar concentrations, leads to a significant potentiation of the swelling-induced ROS production and taurine release (Friis et al. 2008). Although inhibition of the 5-LO system leads to complete impairment of the swelling-induced taurine releases in, e.g. EAT, HeLa, NIH3T3 and C2C12 cells (Lambert and Hoffmann 1993; Lambert and Sepulveda 2000; Lambert 2003; Lambert et al. 2001), it is only in the EAT cells that the 5-LO product, i.e. leukotriene D_4, has been identified and demonstrated to act as a second messenger in the activation of the volume-sensitive taurine efflux pathway (Lambert 1998).

Generation of ROS following osmotic cell swelling has been demonstrated in several cell lines (Friis et al. 2008; Lambert 2003; Diaz-Elizondo et al. 2006; Ortenblad et al. 2003), and it has been shown that a NOX4/p22phox complex constitutes the catalytic core of a volume-sensitive NADPH oxidase in NIH3T3 cells (Friis et al. 2008). Exogenous addition of the reactive oxygen species H_2O_2 has no impact on taurine release under isoosmotic conditions, whereas H_2O_2 potentiates the taurine release once it has been initiated by osmotic cell swelling (Lambert 2007). In this respect, antioxidants impair the swelling-induced taurine release (Lambert 2004). Hence, ROS generated by osmotic cell swelling, are not considered as direct activators of the volume-sensitive taurine transporter *per se* but as competent enhancer of its transport activity. H_2O_2 activates tyrosine kinases and oxidizes essential cysteines in the active sites of protein phosphatases such as the protein tyrosine phosphatase PTP1B (Meng et al. 2002), resulting in loss of enzymatic function. Vanadate, which inhibits protein tyrosine phosphatases, potentiates volume-sensitive taurine release significantly, whereas protein tyrosine kinase inhibitors not only block the swelling-induced taurine release but also eliminate the effect of, e.g. vanadate (Huyer et al. 1997). It is assumed that the increased production of ROS, accompanying osmotic cell swelling, leads to an increased tyrosine phosphorylation state of proteins, involved in the activation/potentiation of the volume-sensitive efflux pathway for organic osmolytes.

Cell swelling has been reported to activate phosphatidylinositol 3-kinases (PI3K) (Wang et al. 2004; Nielsen et al. 2008), resulting in generation of phosphatidylinositol 3,4,5-trisphosphate (PIP_3) from phosphatidylinositol 4,5-bisphosphate (PIP_2) (Vanhaesebroeck et al. 1997), whereas the protein and lipid phosphatase (PTEN) opposes PI3K activity by catalysing the reverse process (Bunney and Katan 2010). PIP_3 generation by PI3K activates downstream effectors, such as protein kinase B (Akt/PKB) and serum-and glucocorticoid-induced protein kinase (SGK), through the phosphoinositide-dependent protein kinase-1 (PDK1) (Wang et al. 2004; Nielsen et al. 2008). Akt/PKB is a negative regulator of the glucose synthase kinase 3 (GSK3), i.e. GSK3 activity is down regulated as a consequence of PI3K activation (Frame and Cohen 2001). Active SGK has been shown to result in increased activity of the volume-sensitive organic osmolyte and anion channel

(VSOAC) as well as several other ion transporters (Wang et al. 2004; Lang et al. 2010), whereas inhibition of GSK3 amongst others increases cell proliferation.

Protein kinase CK2

The constitutively active serine/threonine kinase CK2 is involved in the regulation of diverse processes ranging from cell cycle control, regulation of cellular metabolism, gene expression, protein synthesis as well as modulation of signalling via a broad spectrum of intracellular signalling cascades (reviewed in (Filhol and Cochet 2009; Guerra and Issinger 1999; Meggio and Pinna 2003)). The CK2 holoenzyme is composed of two catalytic subunits (α and/or α') plus two regulatory subunits (β/β) (Thornburg and Lindell 1977), but the catalytic and regulatory subunits also function individually in most cell types (reviewed in (Bibby and Litchfield 2005)). The expression of CK2 is elevated in cancer cells, where CK2 ensures increased cell survival through suppression of apoptosis and increased proliferative capacity (Guerra and Issinger 1999; Sarno and Pinna 2008). CK2 can potentially affect several signal transducers involved in swelling-induced taurine release, including calmodulin (Arrigoni et al. 2004), sPLA$_2$-IIA (Shimoyama et al. 2001), NADPH oxidase (Park et al. 2001) and PTEN (van Diepen et al. 2009; Silva et al. 2008).

The present investigation was initiated to test whether CK2 regulates the active and the passive volume-sensitive taurine transporting systems similarly in two cell types with different expression levels of CK2. We test the effect of specific inhibitors of CK2 on TauT and elements in the swelling-induced signalling cascade that are involved in the activation and modulation of the volume-sensitive taurine efflux pathway.

Materials and methods

Chemicals

Penicillin, streptomycin, Dulbecco's modified Eagle's medium (DMEM), Roswell park Memorial Institute medium (RPMI-1640), foetal calf serum and trypsin were from Invitrogen (Denmark). ^{3}H-labelled taurine and ^{3}H-labelled arachidonic acid were from GE Healthcare (UK). BpV(HOpic) (bisperoxo(bipyridine)oxovanadate) was from Calbiochem (Europe), caboxy-H$_2$DCFDA (5- (and 6-)carboxy -2′,7′-dichloro dihydrofluorescin diacetate) was from Molecular Probes (The Netherlands). All other compounds were from Sigma Chemical Co. (USA). The following stock solutions were prepared in water or DMSO (dimethyl sulfoxide): Carboxy-H$_2$DCFDA (50 mM in DMSO), 2-dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole (DMAT, 10 mM in DMSO), (E)-3-(2,3,4,5-Tetrabromophenyl) acrylic acid (TBCA, 10 mM in DMSO), W-7 (N-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide, 5 mM in H$_2$O), vanadate (Orthovanadate, Na_3VO_4, 20 mM in H$_2$O), Wortmannin (1 mM in DMSO), SB216763 (3-(2,4-Dichlorophenyl)-4-(1-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione, 10 mM in DMSO).

Cell cultures

Experiments were performed with Swiss NIH3T3 mouse fibroblasts (clone 7 obtained from Dr BM Willumsen, Department of Biology, University of Copenhagen) and Ehrlich Lettre ascites tumour cells (ATCC, USA). NIH3T3 and Ehrlich Lettré cells were grown in DMEM and RPMI-1640, respectively, containing 10% heat inactivated FBS and antibiotics (100 U/ml penicillin/0.1 mg/ml streptomycin). Both cell lines were grown at 37°C/5% CO_2/100% humidity and subcultured every 3–4 days by standard trypsination.

Experimental media

Phosphate-buffered saline (PBS) contained 137 mM NaCl, 2.6 mM KCl, 6.5 mM Na_2HPO_4, and 1.5 mM KH_2PO_4. Isoosmotic NaCl medium for NIH3T3 cells contained 159 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4, 1 mM $CaCl_2$, 1 mM $MgSO_4$, and 10 mM *N*-2-hydroxyethyl piperazine-*N*′-2-ethanesulfonic acid (HEPES). Isoosmotic NaCl medium for Ehrlich Lettre cells contained 141 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4, 1 mM $CaCl_2$, 1 mM $MgSO_4$, and 10 mM HEPES. Hypoosmotic NaCl solution was prepared by reduction of the NaCl concentration in the isoosmotic solutions without reducing the other components. Isoosmotic and hypoosmotic *N*-methyl-D-glucamineCl (NMDGCl) and KCl containing media were prepared by substitution of NMDGCl and KCl for NaCl, respectively. pH was in all media adjusted to 7.4.

Western blotting against CK2 α, α' and β

The three subunits of protein kinase CK2 were separated by SDS–PAGE and transferred to PVDF membrane (Bio-Rad, Germany). Proteins were identified by probing the membranes with mouse monoclonal antibodies against CK2 α/α' and $-\beta$ (Calbiochem, USA) and β-actin (Sigma, Denmark). Protein-antibody complexes were visualized by a chemiluminescence detection system following the manufacturer's instructions (CDP-Star, Applied Biosystems, USA). Quantification of subunit expression was carried out using UN-SCAN-IT gel 6.1 gel densitometer analysis software (Silk Scientific Inc., USA).

Estimation of taurine and Na^+ transport kinetics

Taurine uptake was estimated on 80% confluent cells grown in six-well polyethylene plates (9.6 cm^2/well) at room temperature as described previously (Voss et al. 2004). Cells were washed and incubated with isoosmotic media containing ^{3}H-labelled taurine (6.3 × 10^{11} Bq/mmol, 15 or 20 min). For sodium kinetics the extracellular Na^+ concentration was varied between 0 and 150 mM adjusting the osmolarity with NMDGCl. For taurine saturation kinetics cells were incubated with isoosmotic NaCl medium with unlabelled taurine in the concentration from 2 to 100 μM plus ^{3}H-taurine (0.05 μM). Taurine influx was terminated by aspirating the extracellular medium followed by rapid addition and aspiration of ice-cold $MgCl_2$ (2 ml, 100 mM). Cells were subsequently lysed with ethanol (96%), the ethanol blown off and the cellular ^{3}H-taurine activity extracted by addition of ddH_2O (1 ml, 2 h). Cell extracts plus two wash outs were used for estimation of total ^{3}H-taurine (cpm) taken up by the cells at a given time point by β-scintillation counting, (Ultima Gold^{TM}, Perkin Elmer, USA). For protein quantification cells in the sixth well of the polyethylene plates were added isotope-free NaCl medium, proceeded as the wells for isotope uptake before wash outs, hydrolysed (NaOH, 24 h) and the protein contents (mg protein per well) estimated by the Lowry method (Lowry et al. 1951) using BSA as standard. ^{3}H-taurine influx for sodium kinetics (cpm mg protein^{-1} per 15 min) was plotted versus the extracellular Na^+ concentration ($[Na^+]$) and the curves fitted to a Hill type equation: $Y = (V_{max}\cdot[Na^+]^n)/((K_m)^n + [Na^+]^n)$. ^{3}H-taurine influx for taurine kinetics (cpm mg protein^{-1} per 20 min) was plotted versus the extracellular taurine concentration ([taurine]) and fitted to a Michaelis–Menten equation: $Y = (V_{max}\cdot[\text{taurine}])/((K_m) + [\text{taurine}])$. V_{max} is the maximal taurine uptake, K_m is the substrate (Na^+/taurine) concentration required for half maximal taurine uptake and n is the number of Na^+ ions required for initiation of the uptake of one taurine.

Estimation of taurine efflux

Cells were grown as described for estimation of taurine and Na^+ transport kinetics. Each well in the six-well polyethylene plates represented an experimental setup. Cells were pre-loaded for 2 h in growth medium containing ^{3}H-taurine (2 μCi/well, 2 h). The medium was aspirated at the end of the pre-incubation period, the cells washed three times and left with 1 ml isoosmotic NaCl medium. The taurine efflux was executed by sampling of the extracellular medium with 2 min interval. Samples were drawn for 30 min with a shift in tonicity at 8 min. Cells were lysed at the end of the efflux experiment by addition of NaOH (1 ml, 1 M, 1 h). The total ^{3}H-taurine in the cell system was estimated as the sum of ^{3}H-activity in all the efflux samples, the NaOH lysate plus two wash-outs with ddH_2O. For each experimental situation, i.e. well, the natural logarithm to the fraction of ^{3}H-activity remaining in the cells was calculated and plotted versus time. The fractional rate constant for the taurine efflux (min^{-1}) at time (t) was estimated from the logarithmic (ln) plot as the slope between the time point and its proceeding time point. Remaining fraction is estimated at time 30 min, i.e. following 22 min of hypotonic exposure.

Estimation of arachidonic acid release

The protocol followed the protocol outlined for taurine efflux with the exception that cells were loaded with ^{3}H-arachidonic acid (3 μCi/well, 24 h) and that 0.1% BSA was included in the efflux medium in order to trap released arachidonic acid. Release of ^{3}H-arachidonic is given as a fractional release of the ^{3}H-arachidonic acid pool (% min^{-1}).

Cell volume measurements

Absolute cell volumes were estimated by electronic cell sizing in a Coulter Multiziser III (100 μm orifice). Cells (80% confluence) were trypsinated, pelleted by centrifugation (700g, 1 min) and resuspended in isoosmotic NaCl medium for 5 min at 37°C. For estimation of the absolute cell volume, an aliquot was transferred to experimental, filtered media (final cell density ≈ 90,000 cells/ml) and cell volume distribution curves recorded within the initial 5 min (3–4 estimations per minute). Absolute cell volumes (10^{-15} l) were obtained from the median of the distribution curves, using latex beads (diameter 15 μm) for calibration. Rate of volume recovery following hypoosmotic exposure was estimated as the reduction in cell volume within 2 min following maximal cell swelling.

Estimation of ROS production

Cells, grown on pre-treated cover slips, were washed two times with PBS and incubated in serum-free growth medium containing the ROS-sensitive fluorescent probe carboxy-H_2DCFDA (25 μM, 2 h). The cells were subsequently washed with isoosmotic NaCl medium and the cover slip placed in a polystyrene cuvette (50° angle to the excitation light) in isoosmotic or hypoosmotic NaCl medium. ROS estimation was performed on a thermostatic PTI Ratio Master spectrophotometer at 37°C. The excitation and emission wavelengths were 490 nm and 515 nm, respectively, and data were collected every 2 s. for 200 s. The initial increase in fluorescence from 0 to 20 s. was used as an estimation of the ROS production.

Statistical analysis

Data are presented as mean values ± standard error of the mean (SEM). Statistical significance was estimated by paired Student's *t* test. For all statistical evaluations *P* values <0.05 were taken to indicate a significant difference.

Results

Changed substrate kinetics of TauT following CK2 inhibition

Cancer cells have increased levels of CK2 expression compared with their tissues of origin (Guerra and Issinger 1999; Sarno and Pinna 2008), and the present investigation was initiated to test whether increased expression of CK2 affects regulation of taurine transporting systems, and their sensitivity towards CK2 inhibitors. We have previously shown that inhibition of CK2 with DMAT (2-dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole) or TBB (4,5,6,7-tetrabromobenzotriazole) increased taurine uptake via the Na^+-dependent taurine transporter TauT in NIH3T3 cells and that this effect reflected an increased maximal transport rate (V_{max}), an increased affinity of TauT towards Na^+ and a reduction in the Na/taurine stochiometry (Jacobsen et al. 2008). ELA cells express higher levels of all three CK2 subunits compared with NIH3T3 cells (Fig. 1) and Table 1 compares the effect of CK2 inhibition in these two cell lines. We have used TBCA ((E)-3-(2,3,4,5-Tetrabromophenyl) acrylic acid) as CK2 inhibitor, which is a TBB derivative and has been shown to be a more specific and potent CK2 inhibitor compared with TBB and DMAT (Pagano et al. 2008, 2007). From Table 1 it is seen that even though the Na/taurine stoichiometry in NIH3T3 cells is higher than in ELA cells (2.6 vs. 1.8), inhibition of CK2 with TBCA affects NIH3T3 and ELA cells similarly, i.e. V_{max} and K_m for taurine are increased, the Na:taurine stoichiometry is reduced, whereas K_m for sodium is unaffected or slightly reduced. The effect of DMAT on TauT kinetics, taken as a whole, is similar to the effect of TBCA, with the exception, that the reduction in K_m for sodium is more pronounced in NIH3T3 cells exposed to DMAT (Jacobsen et al. 2008).

Reduced taurine release following CK2 inhibition

To test whether CK2 modulates taurine release in parallel to taurine uptake, we investigated the effect of CK2 inhibition on taurine release under isoosmotic as well as hypoosmotic conditions in NIH3T3 and ELA cells. In agreement with previous findings (Lambert 2007), it is seen

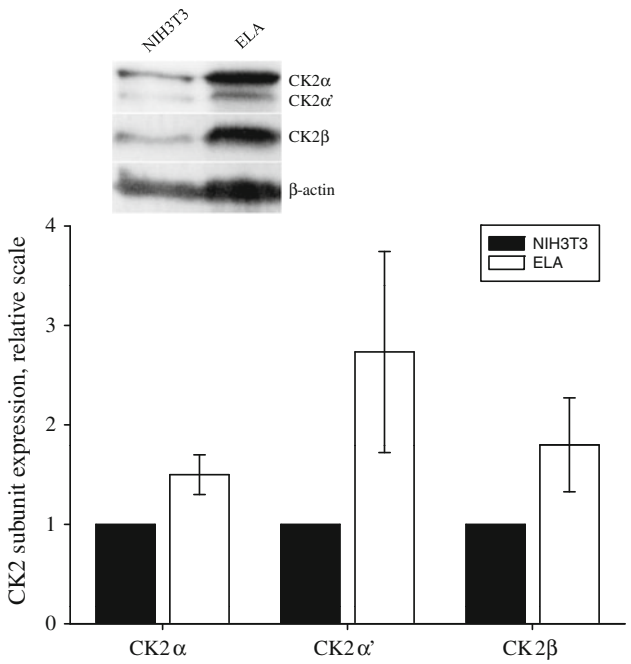

Fig. 1 ELA cells express higher levels of CK2 compared with NIH3T3 cells. Western Blots were prepared and quantified as described in "Materials and methods". Insert: representative western-blot comparing NIH3T3 and ELA cells expression levels of CK2 subunits using *β*-actin as loading control. *Bar diagram* CK2 subunit expression was for each sample normalizes to *β*-actin. Expression levels of CK2 subunits in ELA cells are given relative to expression levels in NIH3T3 cells. Values are given as mean ± SEM and represent three sets of experiments

from Fig. 2 that taurine release from both cell types, shown as the fractional rate constant, is low under isoosmotic conditions, increases significantly following hypoosmotic exposure and reaches a maximal value 2–4 min following the osmotic challenge, where later it gradually returns towards the initial value. The fractional release rate in ELA reaches a maximum under hypotonic conditions, which is 1.5 fold higher than in NIH3T3 cells [0.07 ± 0.002 min^{-1} ($n = 5$) vs. 0.05 ± 0.007 min^{-1} ($n = 11$)]. Taurine efflux is defined as the fractional release rate times the cellular taurine pool. As ELA cells, in addition to the higher release rates (Table 1), have a greater cellular taurine concentration than NIH3T3 cells [17 mM vs. 10 mM (Villumsen et al. 2010; Moran et al. 1997)]; this cell lines have greater taurine efflux under isoosmotic as well as hypoosmotic conditions compared with NIH3T3 cells.

CK2 inhibition by TBCA leads to significant reduction of the rate constant for taurine release under isoosmotic condition in NIH3T3 and ELA cells compared with untreated control cells (Table 1). However, inhibition of CK2 with TBCA in NIH3T3 and ELA cells does not, in spite of the reduced isoosmotic release rate; increased V_{max} and reduced stoichiometry for TauT (Table 1), result in increased long term accumulation of ^{3}H-taurine (24 h,

Table 1 Effect of CK2 inhibition on TauT kinetics (maximal transport capacity, Na/taurine stoichiometry and affinity towards Na^+), taurine release under isoosmotic conditions and long-term taurine accumulation in NIH3T3 and ELA cells

Cell type	Vmax (per cent of control)	Km for taurine (μM)	Km for Na (mM)	Na/taurine stoichiometri	Isoosmotic release (per cent of control)	^{3}H-taurine load (cpm/mg protein)
NIH3T3						
Control	100	8 ± 1.4 (3)	90 ± 8 (6)	2.6 ± 0.2	100	$1.99 \times 10^6 \pm 0.57 \times 10^6$ (3)
TBCA	148 ± 13 (3)*	13 ± 1.4 (3)*	79 ± 12 (5)	2.1 ± 0.3 (5)*	66 ± 11 (5)*	$1.91 \times 10^6 \pm 0.8 \times 10^6$ (3)
ELA						
Control	100	9.7 ± 1.1 (3)	68 ± 4 (6)	1.8 ± 0.1 (6)	100	$2.09 \times 10^6 \pm 0.20 \times 10^6$ (4)
TBCA	171 ± 31 (5)*	20 ± 3 (3)*	62 ± 4 (5)	1.6 ± 0.2 (5)*	66 ± 14 (5)*	$1.86 \times 10^6 \pm 0.15 \times 10^6$ (4)

Taurin influx was estimated from the ^{3}H-taurine uptake within 15 (Na^+ kinetics) or 20 min (taurine kinetics) in control cells and TBCA treated cells (5 μM, 2 h pre-incubation and during experiment). Efflux experiments were conducted and parameters estimated as described in "Materials and methods" and legend to Fig. 2. V_{max} values are given relative to control cells (ELA: 7 ± 1.5 nmol/mg protein•20 min^{-1}; NIH3T3: 6 ± 0.1 nmol/mg protein 20 min^{-1}, $n = 3$). Isoosmotic release were estimated as the average release rates at time 6–8 min and given relative to control (NIH3T3: $1.6 \times 10^{-3} \pm 2.0 \times 10^{-4}$ min^{-1}, $n = 11$; ELA: $1.5 \times 10^{-3} \pm 2.7 \times 10^{-4}$ min^{-1}, $n = 5$). ^{3}H-taurine load is estimated as total accumulated activity following 24 h of incubation with or without TBCA. Values are given as mean ± SEM. The number of experiments is indicated in the parenthesis. *Significant difference from control values

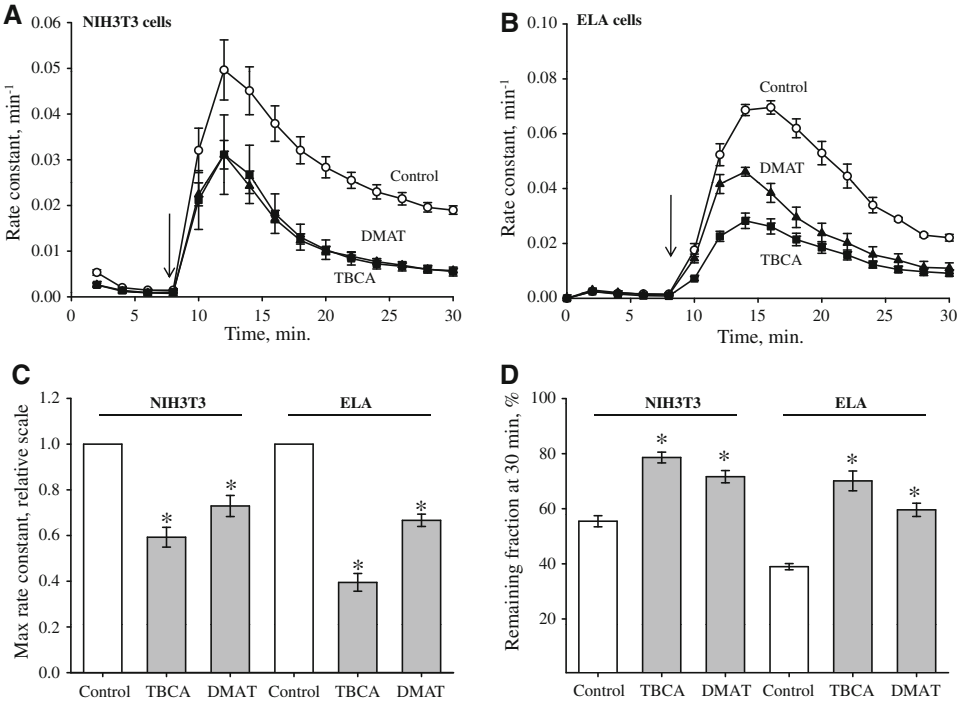

Fig. 2 CK2 inhibition reduces taurine release in NIH3T3 and ELA cells. Cells, grown at 80% confluence, were pre-incubated with ^{3}H-taurine for 2 h, washed in tracer-free, isoosmotic NaCl medium (NIH3T3 cells: 335 mOsm l^{-1}, ELA cells 300 mOsm l^{-1}) before being exposed to isoosmotic NaCl (0–8 min) and hypoosmotic NaCl medium (200 mOsm l^{-1}, 8–30 min). The taurine release was estimated in 2-min intervals and expressed as fractional release rate constants (min^{-1}). **a**, **b** Fractional taurine release rate constants as function of time in NIH3T3 and ELA Control cells (*open symbols*) and cells exposed to TBCA (5 μM, *closed triangles*) or DMAT (5 μM, *closed squares*). TBCA and DMAT were present from 2 h before and during the efflux experiment. *Arrow* indicates the reduction in osmolarity. **c** Maximal rate constant under hypoosmotic conditions in the absence (*open bars*) or presence of TBCA/DMAT (*grey bars*). Values are given relative to Control (NIH3T3: 0.05 ± 0.007 min^{-1}; ELA: 0.07 ± 0.002 min^{-1}). **d** Remaining taurine fraction was estimated at time 30 min., i.e. after 22 min hypoosmotic exposure in the absence (*open bars*) or presence of TBCA/DMAT (*grey bars*). All values are given as mean ± SEM. Values for NIH3T3 cells represents 11, 5 and 6 sets of experiments for Control, TBCA and DMAT, respectively. Values for ELA cells represent five sets of experiments. *Asterisk* indicates significant difference from control values

Table 1). This inconsistency reflects the accompanying decrease in TauT taurine affinity resulting in unchanged actual uptake rate under the experimental conditions where the substrate concentration is 0.05 μM, i.e. it is significantly lower than K_m for taurine (Table 1). It is assumed that under more physiological conditions, where substrate concentration is 100 μM (Jacobsen and Smith 1968), the increased V_{max} will dominate and taurine uptake increase, following CK2 inhibition.

From Fig. 2 it is seen that CK2 inhibition by TBCA and DMAT reduces the maximal fractional rate constants significantly under hypoosmotic conditions in NIH3T3 as well as ELA cells (Fig. 2a–c), and the remaining cellular fraction of ^{3}H-taurine in both cell types, estimated 22 min after hypoosmotic exposure, is increased by CK2 inhibition when compared with untreated control cells (Fig. 2d). The decreased fractional release rate following CK2 inhibition reflects reduced open-probability of the volume-sensitive taurine efflux system. Thus, the higher fractional release rate in ELA cells following osmotic swelling, compared with NIH3T3, could be the result of greater CK2 expression (see Fig. 1).

Indirect, CK2-mediated effect on the inactivation of the volume-sensitive taurine release

Taking the volume-sensitivity of the swelling-induced taurine release into consideration, the reduced fractional release rates following incubation with DMAT and TBCA (Fig. 2) could reflect an accelerated RVD response caused by an increased ion loss via volume-sensitive ion channels/transporters, and hence an accelerated inactivation of the volume-sensitive taurine release pathway. From Fig. 3a it is seen that TBCA and DMAT accelerate the RVD response in NIH3T3 cells following exposure to hypoosmotic (200 mOsm) NMDGCl medium. Exposing cells to a greater hypoosmotic challenge (150 mOsm NMDG ringer), in order to accelerate the RVD even further, results in near depletion of ^{3}H-taurine (Fig. 3b). However, even under

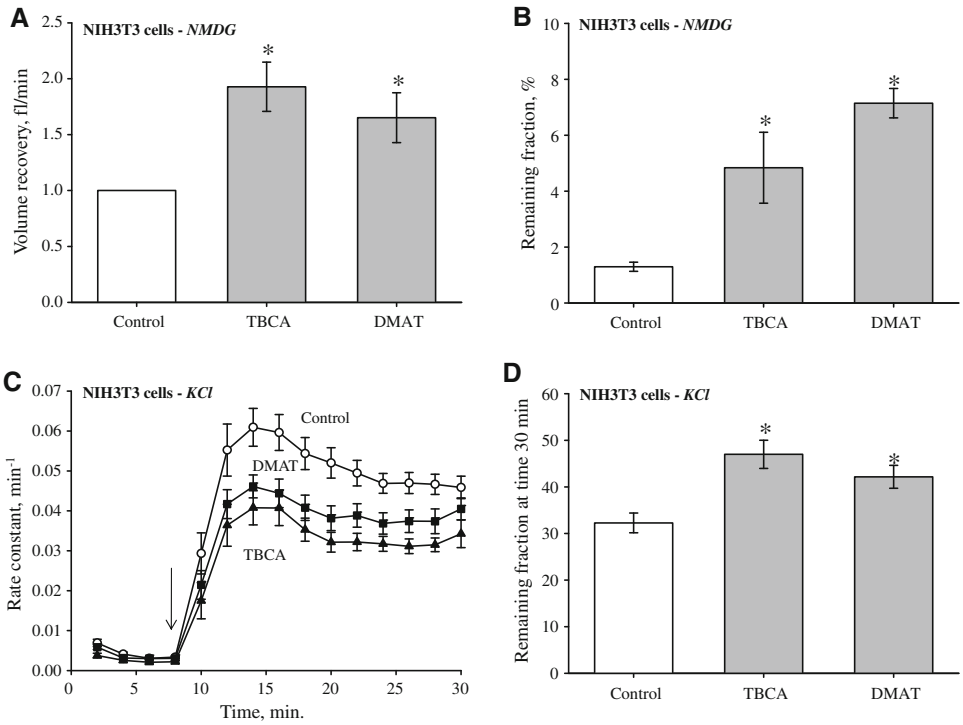

Fig. 3 Accelerated volume regulation does not explain decreased fractional taurine release rates following CK2 inhibition. Experiments were conducted on NIH3T3 cells grown at 80% confluence. Volume recovery measurements were conducted as described in "Materials and methods" using NMDGCl medium. Taurine efflux was conducted as indicated in the legend to Fig. 2 using NaCl and KCl medium. TBCA or DMAT (5 μM) when present, was added 2 h prior to and during the experiment. *Symbols* are defined in legend to Fig. 2. **a** Volume recovery (fl min^{-1}) in cells exposed to hypoosmotic (200 mOsm) NMDGCl medium in the absence or presence of TBCA or DMAT and represents 3 and 5 sets of paired experiments for DMAT and TBCA, respectively. **b** Remaining taurine fraction following 22 min exposure to hypoosmotic (150 mOsm) NMDGCl medium and represents 9, 4 and 5 experiments for Control, TBCA and DMAT, respectively. **c** Fractional taurine release rate constants in KCl ringer as function of time in Control cells and cells exposed to TBCA or DMAT. *Arrow* indicates reduction in osmolarity from 335 to 200 mOsm. Number of experiments is 7, 4 and 5 for Control, TBCA and DMAT, respectively. **d** Remaining taurine fraction was estimated at time 30 min., i.e. after 22 min' hypoosmotic exposure in the absence or presence of TBCA or DMAT. Number of experiments is 13, 8 and 9 for Control, TBCA and DMAT, respectively. All values are given as mean ± SEM. *Asterisk* indicates significant difference from Control

these conditions, TBCA and DMAT increase the remaining fraction significantly (Fig. 3b).

In order to eliminate the possibility of indirect effect of the accelerated RVD of CK2 inhibition on the taurine release rates and remaining fraction we exposed NIH3T3 cells to hypoosmotic (200 mOsm) KCl medium, i.e. a condition where the normal RVD response is prevented by the high extracellular K^+ concentration (Sarkadi et al. 1984). From Fig. 3 it is seen that the fractional release rate for taurine in KCl is increased as a result of osmotic swelling; however, as the RVD response is prevented, the increased fractional release rate is maintained during the experiment. The remaining ^{3}H-taurine fraction is reduced by 40% compared with cells exposed to hypoosmotic NaCl, i.e. from 56 (Fig. 2d) to 32% (Fig. 3d). Inhibition of CK2 in NIH3T3 cells under the non RVD-permissive conditions results in decreased taurine release rates (Fig. 3c) and increased remaining ^{3}H-taurine fraction (Fig. 3d) as seen under RVD-permissive conditions (Fig. 2). This is taken to indicate that the reduced fractional taurine release rate under RVD-permissive conditions is not indirectly caused by the accelerated RVD response by TBCA and DMAT. Thus, CK2 is modulating signalling transducers from cell swelling to activation of the taurine release channel, or the channel directly.

Set-point

Swelling-induced taurine release from NIH3T3 cells is previously shown to increase with the osmotic challenge (Lambert 2003). To test whether CK2 affects the initiation of the signalling pathway via a sensor of cell swelling, i.e. by changing the volume set-point for the volume-sensitive taurine release system, we followed taurine release with time from NIH3T3 cells after exposure to decreasing osmolarities (isoosmotic: 320 mOsm, hypoosmotic: 285–195 mOsm). The maximal rate constant, obtained under the hypoosmotic condition, was used as an indication of the volume-sensitive taurine release. From Fig. 4 it is seen that there is a significant increase in the taurine release rate when cells are exposed to hypoosmotic NaCl media at 218 mOsm or below. The volume-sensitive taurine release in TBCA-treated cells is like the release in the untreated control cells significantly activated at 218 mOsm and lower osmolarities (Fig. 4), indicating that CK2 inhibition does not affect the osmolarity needed for activation of the volume-sensitive taurine release.

Volume-sensitive PLA_2 activity

Activation of the intracellular Ca^{2+}-independent PLA_2 ($iPLA_2$) and secretory PLA_2 ($sPLA_2$) is an upstream event in the activation of swelling-induced taurine release pathway in NIH3T3 cells (Lambert and Pedersen 2006; Pedersen et al. 2006), and it has also been demonstrated that

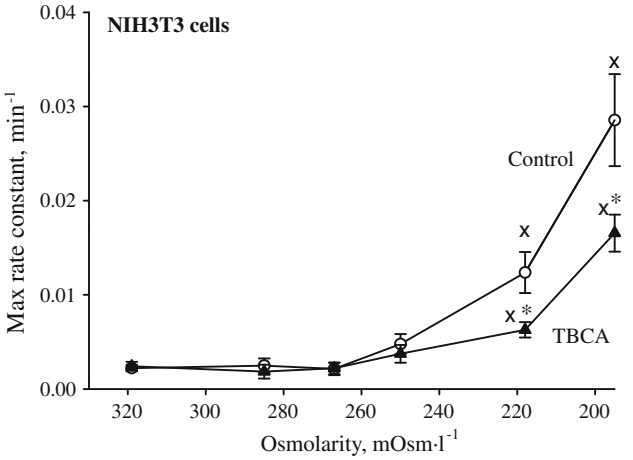

Fig. 4 CK2 inhibition does not affect the set-point for activation of the volume-sensitive taurine release. NIH3T3 cells, grown at 80% confluence, were pre-incubated with ^{3}H-taurine for 2 h. The cells were exposed to isoosmotic NaCl medium (335 mOsm l^{-1}, 0–8 min) and hypoosmotic NaCl medium (300 to 200 mOsm l^{-1}, 8–30 min). The fractional taurine release was estimated in 2-min intervals and expressed as fractional release rates (min^{-1}). The maximum fractional release rate was determined for each osmolarity in the absence (Control, *open symbols*) or presence of TBCA (5 μM, *closed symbols*) and plotted against the measured values for the extracellular osmolarity (depression of freeze-point, Knauer osmometer automatic). *x* indicates significant difference compared with isoosmotic values, *asterisk* indicates significant difference from Control cells at equivalent osmolarity. Data are given as average values ± SEM and represents four sets of experiments

hypoosmotic cell swelling in the NIH3T3 cells is associated with loss of arachidonic acid from the nucleus which is inhibited by the $iPLA_2$ specific inhibitor bromoenol lactone (BEL; Lambert and Pedersen 2006; Pedersen et al. 2006). The catalytic activity of $iPLA_2$ ($iPLA_2\beta$) is inhibited by calmodulin interaction (Jenkins et al. 2001), and CK2 mediated phosphorylation is reported to inhibit calmodulin interaction with other proteins (Arrigoni et al. 2004). In the case of NIH3T3 cells it has previously been shown that exposure to the calmodulin antagonist W-7 potentiates the activation of the swelling-induced taurine release pathway (Lambert 2004). This is confirmed in Fig. 5, where it is shown that W-7 increases the maximal taurine release rate constant 1.56 ± 0.14 fold ($n = 6$). From Fig. 5b, it is seen that the relative reduction in the maximal release rate constants, induced by TBCA or DMAT, is unchanged in the presence of W-7, indicating that the inhibition of CK2 does not affect the taurine release through calmodulin.

$iPLA_2$ has a potential CK2 phosphorylation site (Mancuso et al. 2000), and CK2 phosphorylation of $sPLA_2$-IIA has been shown to stimulate the PLA_2 activity in vitro (Shimoyama et al. 2001). Inhibition of $iPLA_2$ with BEL and $sPLA_2$ with manoalide has previously been shown to reduce the release of arachidonic acid to the extracellular compartment under hypoosmotic conditions in NIH3T3 cells

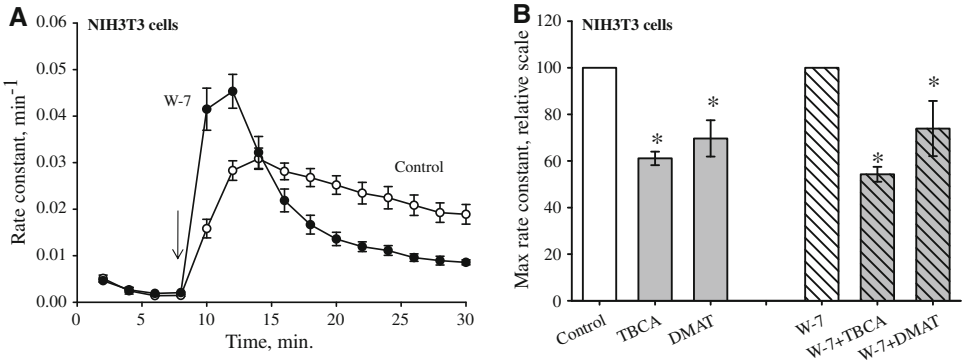

Fig. 5 Reduction in volume-sensitive taurine release following CK2 inhibition does not involve calmodulin. NIH3T3 cells were prepared and the taurine efflux conducted as indicated in the legend for Fig. 2. DMAT (5 μM) and TBCA (5 μM), when present, were added 2 h prior to and present during the efflux experiment. The calmodulin inhibitor W-7 (50 μM) was only present during the efflux experiment. **a** Time trace of fractional release rates in Control cells (*open symbols*) and cells exposed to W-7 (*closed symbols*). Reduction in osmolarity (335 to 200 mOsm l^{-1}) is indicated by the *arrow*. **b** Maximal fractional release constant for taurine in hypoosmotic NaCl medium in the absence (Control, *white bar*) or presence of TBCA/DMAT (*grey bars*) and/or W-7 (*hatched bars*). Values are given relative to Control (0.031 ± 0.002 min^{-1}) or W-7 (0.047 ± 0.004 min^{-1}). Data represent 5, 4, 4 and 5 sets of paired experiments for TBCA, DMAT, W-7 plus TBCA and W-7 plus DMAT, respectively. **c** Inactivation of the taurine release following 22 min' hypoosmotic exposure in the absence (Control, *white bar*) or presence of TBCA/DMAT (*grey bars*) and/or W-7 (*hatched bars*). Data represent 6, 6, 5, 5, 4 and 5 sets of experiments for Control, W-7, TBCA, W-7 plusTBCA, DMAT, and W-7 plus DMAT, respectively. *Asterisk* indicates significant difference from Control. #Significant difference from W-7 treated cells

(Pedersen et al. 2006). From Fig. 6a it is seen that there is a significant increase in released ^{3}H-activity from NIH3T3 cells, pre-loaded with ^{3}H-labelled arachidonic acid, within 4 min following hypoosmotic exposure (200 mOsm). Using the release of ^{3}H-arachidonic acid at time 6–8 min (isoosmotic condition) and the average release at time 12–20 min (hypoosmotic condition), it is estimated that osmotic swelling increases the fractional release of arachidonic acid from 0.03 to 0.06% (Fig. 6a). The swelling-induced arachidonic acid release is unaffected by DMAT (Fig. 6b, d), whereas it is significantly reduced in the presence of TBCA (Fig. 5c, d). It is plausible that the effect of TBCA on arachidonic acid release is not directly mediated by CK2 as DMAT exposure does not result in similar results. Such a significant reduction in arachidonic acid mobilization would be expected to result in severe retardation of the general RVD response, including potassium and chloride release, which on the contrary was stimulated following treatment with TBCA (Fig. 3a).

Swelling-induced ROS production and ROS mediated potentiation of the volume-sensitive taurine release

NIH3T3 cells express the regulatory NADPH oxidase subunits $p67^{phox}$, $p47^{phox}$, $p22^{phox}$ and the catalytic subunits NOXO1, NOX4 and DUOX2 with NOX4/$p22^{phox}$ being sufficient for generation of ROS (Friis et al. 2008). $p47^{phox}$ does not seem to be directly required for the swelling-induced potentiation of the ROS production in NIH3T3 cells (Friis et al. 2008) although the role of $p47^{phox}$ and $p67^{phox}$ is unclear. On the other hand, CK2 has been shown to phosphorylate $p47^{phox}$ thereby potentially inhibiting the activation of the NADPH-oxidase (Park et al. 2001), giving CK2 a putative role in regulation of ROS production in the NIH3T3 cells. To test the effect of TBCA and DMAT on the swelling-induced increase in the ROS production, we used the ROS-sensitive fluorescent probe carboxy-H_2DCFDA. In agreement with previously published data (Lambert 2007; Friis et al. 2008) it is seen in Fig. 7a, b, that exposure to hypoosmotic media (200 mOsm) results in a significant increase in ROS production in NIH3T3 cells. Using the initial increase in fluorescence intensity following hypoosmotic exposure as an estimate of the ROS production it is seen from Fig. 7b that exposure to TBCA and DMAT has no effect on the ROS production under isoosmotic conditions, whereas inhibition of CK2 has a tendency to potentiate the swelling-induced increase in the ROS production. It is estimated that exposure to DMAT potentiates the swelling-induced ROS production 1.6 fold (Fig. 7b). However, an increase ROS production would lead to a potentiation of the swelling-induced taurine release and not, as observed, a decrease in the release (see Fig. 2).

To test whether the reduced taurine release seen in CK2 inhibited cells could reflect a reduced sensibility towards ROS, we followed the effect of 0.5 mM H_2O_2 on the taurine release under isoosmotic and hypoosmotic conditions (200 mOsm) in control cells and cells treated with TBCA. In agreement with previously published data (Lambert 2003), it is seen from Fig. 7c that H_2O_2 exposure does not affect the taurine release rates under isoosmotic conditions, whereas it significantly potentiates the release rates following hypoosmotic exposure. Exposure to TBCA leads to

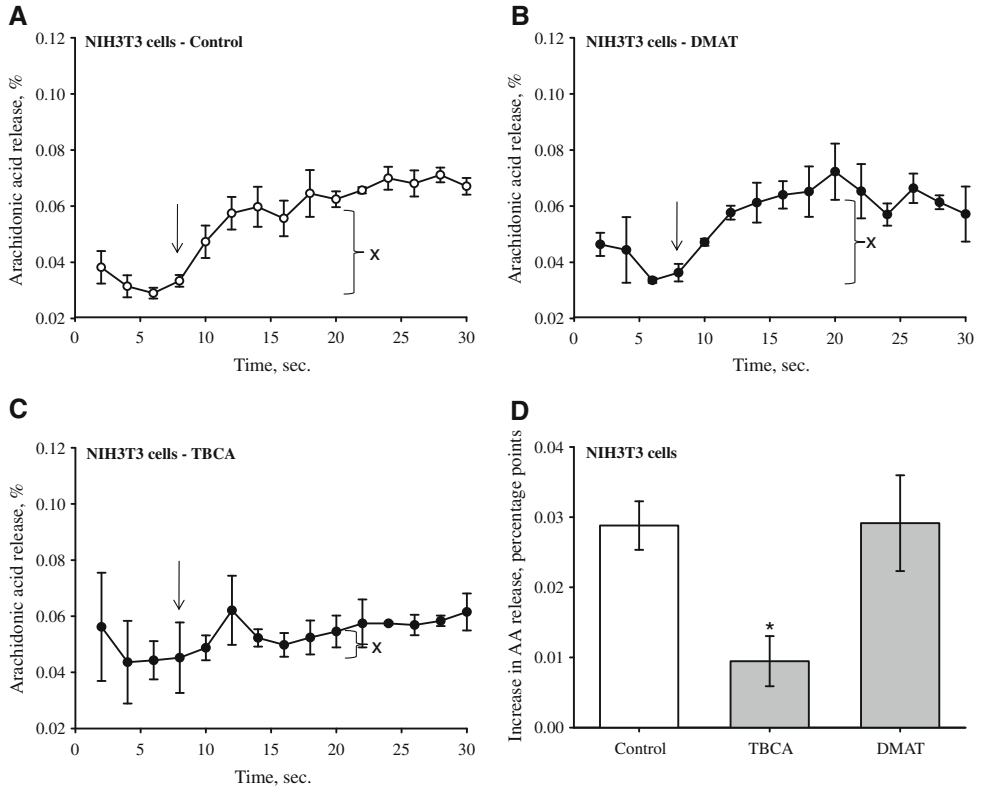

Fig. 6 Effect of CK2 inhibition on swelling-induced arachidonic acid release. NIH3T3 cells, grown at 80% confluence, were pre-incubated with ^{3}H-labelled arachidonic acid for 24 h before the experiment. DMAT (5 μM) and TBCA (5 μM) when present were added 2 h prior to and present during the release experiment. The arachidonic acid release experiment was performed by reducing the medium osmolarity (335 to 200 mOsm l^{-1}) and estimating the arachidonic acid release (%) as described in "Materials and methods". **a–c** Time traces of arachidonic acid release from Control cells (**a**), cells exposed to DMAT (**b**) or TBCA (**c**). Reduction in osmolarity is indicated by the *arrow*. *x* represents significant increase in arachidonic acid release compared with isoosmotic conditions. **d** Increase in arachidonic acid release following reduction in osmolarity (*x*), calculated as the difference in release during isoosmolarity (time 6–8 min) to average release during hypoosmolarity (time 12–20 min) and presented as increase in percentage points. *Asterisk* indicates significant difference compared with control

a significant reduction in maximal release rates in the ROS-stimulated cells (Fig. 7c), and it is estimated that the relative reduction in the maximal rate constant in H_2O_2-treated cells is similar to the relative reduction in cells not stimulated by H_2O_2 (Fig. 7d). Hence, the effect of CK2 inhibition on the taurine release under hypoosmotic conditions seems not to involve the ROS mediated regulation.

Effect of CK2 inhibition on tyrosine phosphotases and PI3K/PTEN signalling

Inhibition of protein tyrosine phosphatases with vanadate has been shown to potentiate the maximal release rate constant and delay the inactivation of the volume-sensitive taurine release pathway in NIH3T3 cells, whereas inhibition of protein tyrosine kinases not only inhibits the activation of the efflux pathway but also eliminates the effect of phosphatase inhibition (Lambert 2003). Data in Fig. 8 confirm the effect of vanadate on taurine release under hypoosmotic conditions in NIH3T3 cells. The potentiating effect of vanadate is completely lifted by TBCA, and there is no significant difference in the maximal release rates under hypoosmotic conditions in TBCA-treated and in TBCA-plus vanadate-treated cells (Fig. 8). The effect of TBCA on the maximal rate constant is similar to the tyrosine kinase inhibitor genistein (Lambert 2004), i.e. there is no significant difference in maximal rate constants for control and vanadate-treated cells in the presence of TBCA ($P > 0.2, n = 3$). This is taken to indicate that CK2 modulates the tyrosine phosphorylation state of the taurine release pathway or a signalling component leading to its activation.

CK2 is known to reduce the activity of PTEN, and to increase PTEN stability as well as membrane localization (van Diepen et al. 2009; Silva et al. 2008). PTEN antagonises PI3K signalling. Furthermore, PTEN is inhibited by reactive oxygene species (Silva et al. 2008) and possibly by vanadium compounds (Lai et al. 2007; Schmid et al. 2004). Hence, PTEN is a potential downstream signalling transducer in the swelling-induced taurine release pathway. We used the specific PTEN inhibitor HOpic and the PI3K

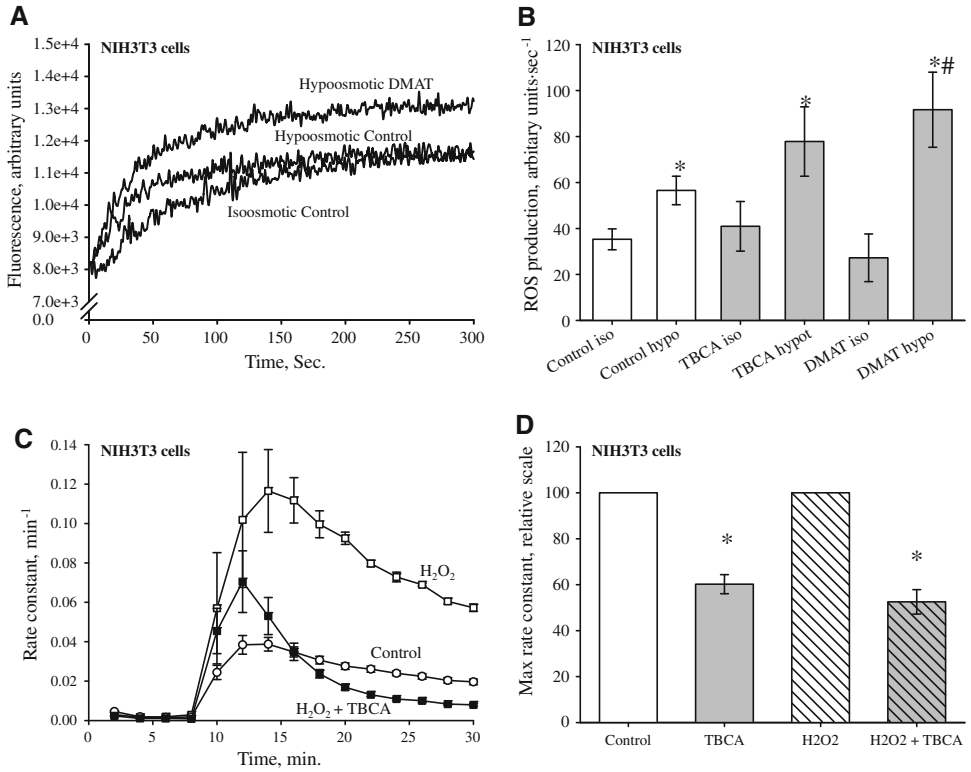

Fig. 7 Effect of CK2 inhibition on the production of, and sensitivity towards, reactive oxygen species. The production of reactive oxygene species and the sensitivity towards H_2O_2 stimulation were estimated in NIH3T3 cells (80% confluence) in the absence (Control) or presence of TBCA or DMAT (5 μM) as described in "Materials and methods". Taurine efflux was conducted as indicated in the legend to Fig. 2. H_2O_2 (0.5 mM) was only present during the efflux experiment. **a** Time traces of fluorescence intensity (arbitrary units) from cells in isoosmotic medium, hypoosmotic medium and cells pre-treated with DMAT in hypoosmotic medium. **b** Initial increase in fluorescence intensity (0–20 s) in isoosmotic and hypoosmotic media in the absence (Control, *white bars*) or presence of TBCA/DMAT (*grey bars*), was used as an estimate of the ROS production. **c** Time trace of fractional release rates in Control cells (*open circles*), cells exposed to H_2O_2 (*open squares*) and cells exposed to H_2O_2 plus TBCA (*closed squares*). Reduction in the osmolarity (335–200 mOsm l^{-1}) is indicated by the *arrow*. **d** Maximal fractional release constant for taurine in hypoosmotic NaCl medium in the absence of TBCA (*white bars*), in the presence of TBCA (*grey bars*), H_2O_2 (*hatched bars*), and TBCA plus H_2O_2 (*grey*, *hatched bars*). Values for **a** and **b** are presented as average ± SEM and represent 11, 9, 5, 4, 5 and 3 experiments for isoosmotic Control, hypoosmotic Control, isoosmotic plus TBCA, hypoosmotic plus TBCA, isoosmotic plus DMAT and hypoosmotic plus DMAT, respectively, *Asterisk* indicates significant different ROS production compared with the isoosmotic Control. #Significant larger ROS production compared with hypoosmotic Control. **c**, **d** Represent 8, 6 and 7 sets of experiments for TBCA, H_2O_2 and H_2O_2 plus TBCA, respectively. Values for **d** are given relative to Control cells (0.030 ± 0.002 min^{-1}) or H_2O_2 treated cells (0.114 ± 0.011 min^{-1}), *Asterisk* indicates significant difference from Control or H_2O_2 treated cells

inhibitor Wortmannin in order to determine whether CK2 regulates the volume-sensitive taurine release via PTEN/PI3K signalling. It is seen in Fig. 8a that inhibition of PTEN and PI3K increases and reduces the swelling-induced taurine release, respectively. Furthermore, TBCA treatment decreases the swelling-induced taurine release even under conditions where PTEN is inhibited (Fig. 8c). The effects of TBCA and Wortmannin on taurine release are not additive, i.e. the decrease in maximum taurine release rate in the presence of Wortmannin plus TBCA is not more pronounced than with TBCA alone (Fig. 8c). These results are taken to indicate that CK2 could modulate signalling downstream to PI3K. However, this modulation does not necessarily involve inactivation of PTEN.

GSK3 is inactivated by Akt/PKB, the activity of which is increased by PI3K following cell swelling (Wang et al. 2004; Nielsen et al. 2008), and GSK3 phosphorylation is in some cases primed by CK2-mediated phosphorylation (Fiol et al. 1987). As TBCA as well as DMAT, in addition to CK2, are reported to inhibit GSK3 (Pagano et al. 2008, 2007), the effect of these inhibitors on the taurine release rates could be (1) a direct effect of CK2 inhibition, (2) an indirect effect of GSK3 inhibition and (3) inhibition of CK2, affecting a priming phosphorylation for GSK3 in conjunction with general inhibition of GSK3 activity. In order to determine whether TBCA and DMAT have these putative off-target effects on the taurine release pathway, we examined the effect of GSK3 inhibition using the specific GSK3 inhibitor SB216763. From Fig. 8b, c it is seen that inhibition of GSK3 with SB216763 results in significantly reduced taurine release rate following hypoosmotic exposure. However, the time trace of taurine

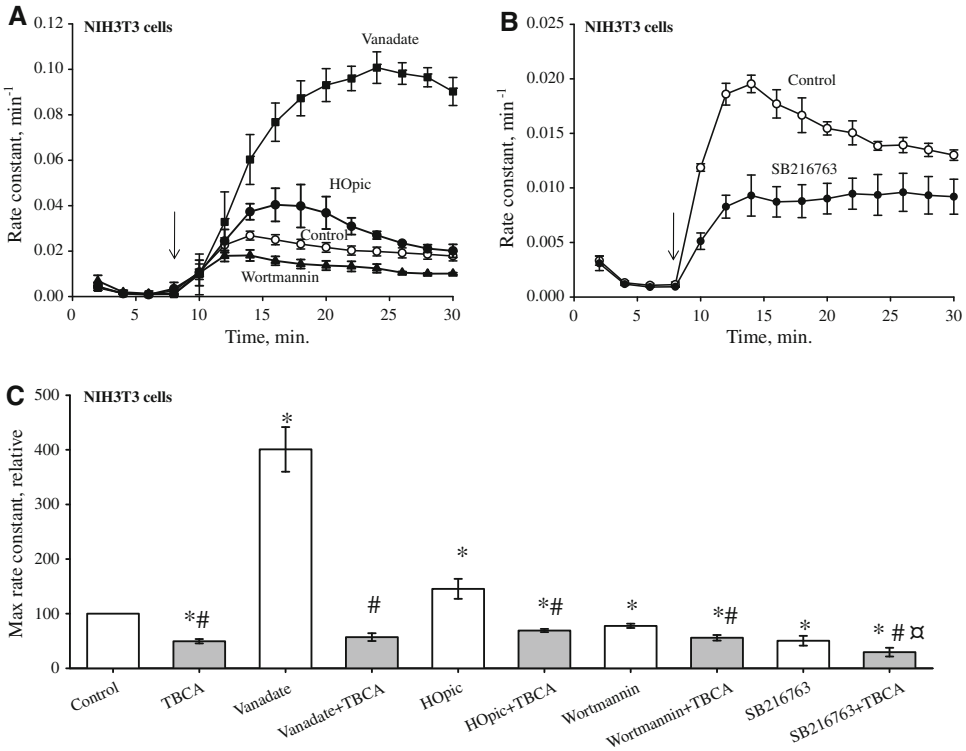

Fig. 8 Potentiation of the volume-sensitive taurine release by vanadate and HOpic in NIH3T3 cells is reduced by CK2 inhibition. NIH3T3 cells were prepared, the taurine efflux conducted and release rates calculated as indicated in legend for Fig. 2. TBCA and SB216763 (5 μM), when present, were added 2 h prior to and present during the efflux experiment. Wortmannin, when present, was added 20 min prior to and during the experiment. Vanadate (50 μM) and HOpic (5 μM) were only present during the efflux experiment. **a** Time trace of fractional release rates in Control cells (*open circles*), cells exposed to vanadate (*closed squares*), HOpic (*closed circles*) and Wortmannin (*closed triangles*). Reduction in osmolarity (335–200 mOsm l^{-1}) is indicated by the *arrow*. **b** Time trace of fractional release rates in Control cells (*open circles*) and cells exposed to SB216763 (*closed circles*). Reduction in osmolarity is indicated by the *arrow*. **c** Maximal fractional release constant for taurine in hypoosmotic NaCl medium in the absence (*white bars*) or presence of TBCA (*grey bars*). Values are given relative to Control cells and the number of paired experiments is: 21 (Control, TBCA), 3 (Vanadate ± TBCA), 4 (HOpic ± TBCA), 3 (Wortmannin ± TBCA), 3 (SB216763 ± TBCA). *Asterisk* indicates significant difference from Control, #Significant effect of TBCA and ¤ indicates additive effect of TBCA

release rates in the presence of SB216763 differs from that of TBCA or DMAT, i.e. there is no inactivation of the taurine release pathway in the presence of SB216763 (compare Figs. 2, 8b). Furthermore, inhibition of GSK3 does not affect the isoosmotic taurine release rates (90 ± 17% of control, $n = 3$), and SB216763 in combination with TBCA has an additive effect on the swelling-induced release rates (Fig. 8c). Together, these data are taken to indicate that GSK3 and CK2 affect the taurine release via separate signalling pathways.

Discussion

CK2 regulates active taurine uptake via TauT and passive taurine release via the volume-sensitive transporter for organic osmolytes

CK2 inhibition results in changed TauT kinetics, i.e. V_{max} and K_m for taurine are increased, the Na:taurine stoichiometry is reduced, whereas K_m for sodium is slightly (TBCA, Table 1) or significantly [DMAT, (Jacobsen et al. 2008)] reduced. The response towards CK2 inhibition on the taurine uptake kinetics is similar in NIH3T3 and ELA cells, the latter of which express increased levels of regulatory and catalytic subunits of CK2. If CK2 expression levels correlate with TauT kinetics, we would expect that the increased expression of CK2 in ELA cells should result in TauT kinetics opposite to that seen in CK2-inhibited cells. This is not the case, and we currently assume that normal function of TauT depends on CK2 activity, but is unaffected by changed CK2 expression levels.

Inhibition of CK2 limits the open-probability of the taurine release pathway in NIH3T3 and ELA cells under isoosmotic as well as hypoosmotic conditions (Fig. 2; Table 1). The taurine transporters involved in taurine efflux under isoosmotic and hypoosmotic conditions have not yet been identified. However, the taurine efflux differs from taurine uptake by TauT as it has different pH profile and is sodium independent (Lambert 2004; Lambert and

Hoffmann 1993). Several translation products of TauT have been demonstrated in various cell types (Jacobsen et al. 2008; Poulsen et al. 2002; Voss et al. 2004). TauT contributes to isoosmotic taurine release, whereas it does not contribute to swelling-induced taurine release (Lambert 2004; Voss et al. 2004). The swelling-induced taurine efflux system is, in contrast to the isoosmotic taurine release system, sensitive towards inhibition by DIDS (Villumsen et al. 2010), and stimulation by ROS (Lambert 2003), indicating that swelling-induced taurine release is independent of TauT. Isoosmotic and hypoosmotic release are, however, both sensitive to CK2 inhibition.

Our observations also indicate that despite the reduced open-probability of the taurine leak pathways following inhibition of CK2, the overall volume restoration following osmotic cell swelling is accelerated. The K^+ permeability is rate limiting the RVD response in NIH3T3 cells following osmotic cell swelling (Lambert 2007), i.e. an increase in the K^+ permeability will accelerate not only loss of KCl and hence volume restoration but also the inactivation of the volume-sensitive taurine release pathway. CK2 plus the protein phosphatase 2A (PP2A) co-associate with small conductance, calcium-activated potassium channels (SK), and it has been shown that CK2-phosphorylation of CaM inactivates the SK-channel by reducing the Ca^{2+} sensitivity of the system, whereas the activity of PP2A increases the Ca^{2+} sensitivity (Bildl et al. 2004; Allen et al. 2007). Whether CK2 modulates the RVD response through volume-sensitive (TASK2) or SK-channels, is to be determined. However, inhibition of CK2 still limits taurine release even when the RVD response is prohibited by high extracellular K^+, indicating that the effect of the CK2 inhibitors on the fractional release rates are caused by direct modulation of the taurine release pathways or signalling transducers controlling open possibility of the pathways.

CK2 regulates taurine release downstream to known volume-sensitive signal transducer

CK2 inhibition does not seem to interfere with the set-point for taurine release, mobilization of arachidonic acid by PLA_2 or generation of/sensitivity towards ROS. ROS are known to inhibit multiple phosphatases including protein tyrosine phosphatases (Lee et al. 1998), as well as protein serine-threonine phosphatases (Rao and Clayton 2002). Previous studies have shown that the broad range tyrosine phosphatase inhibitor vanadate potentiates the swelling-induced release of taurine, and inhibition of tyrosine kinases with genistein not only prevents the activation of the volume-sensitive taurine release pathway but also suppress the vanadate-induced potentiation in NIH3T3 cells (Lambert 2003). Thus, tyrosine kinase activity is a prerequisite for activation of the volume-sensitive pathway for organic osmolytes in NIH3T3 cells. Inhibition by TBCA completely lifts the effect of vanadate on the swelling-induced taurine release (Fig. 8). This response is similar to the effect of genistein (Lambert 2004), indicating that CK2 could affect the activity of a tyrosine kinase, either directly or indirectly.

Inhibition of PI3K results in decrease taurine release following cell swelling and as PTEN antagonizes PI3K signalling, the increased taurine release following inhibition of PTEN is in line with the involvement of PI3K/PTEN in the swelling-induced activation of VSOAC (Wang et al. 2004). As CK2 is known to inhibit PTEN (Silva et al. 2008) we expected that inhibition of PTEN would render CK2 inhibition ineffective. However, exposure to TBCA results in decreased taurine release rates even when PTEN is inhibited, indicating that CK2 does not regulate taurine release through PTEN.

We demonstrate that inhibition of GSK3 results in reduced taurine release rate and that inhibition of CK2 is additive with inhibition of GSK3. This indicates that both of these kinases are positive regulators of the swelling-induced taurine release pathway and that they are involved in parallel signalling pathways. Akt/PKB is known to phosphorylate and inactivate GSK3β in response to insulin or PI3K activation and as GSK3 is a positive regulator of the taurine release pathway, this would seem to be in conflict with the positive effect of inhibiting PI3K on taurine release. However, Akt/PKB phosphorylation inactivates only a specific subpopulation of GSK3β that requires substrates which are primed through phosphorylation (Weston and Davis 2001). Thus, priming of GSK3 targets by CK2, would in this case not be necessary for GSK3β activity, explaining why inhibition of GSK3 and CK2 are additive even in the presence of activated Akt/PKB.

Figure 9 summarises our present knowledge of swelling-induced activation of the release pathway for the organic osmolyte taurine. At least three separate signalling cascades are activated in parallel by unknown sensors of cell volume, and activation of PLA_2, NADPH oxidase and PI3K can be considered upstream events. Cross-talk appears between the three pathways, i.e. ROS potentiate the signalling cascades initiated by PLA_2 and PI3K, whereas lysophospholipids, generated by PLA_2 stimulates the NADPH oxidase. The exact role of PDK1 and SGK in volume-sensitive taurine release requires further experiments. Inhibition of CK2 has no effect on PLA_2 activity and ROS production by NADPH oxidase, whereas it lifts the effect of PTEN and PTP inhibition. It is suggested that CK2 regulates the taurine release downstream to PLA_2, PTP and PI3K. The volume-sensitive efflux pathway for taurine has to be cloned in order to determine CK2

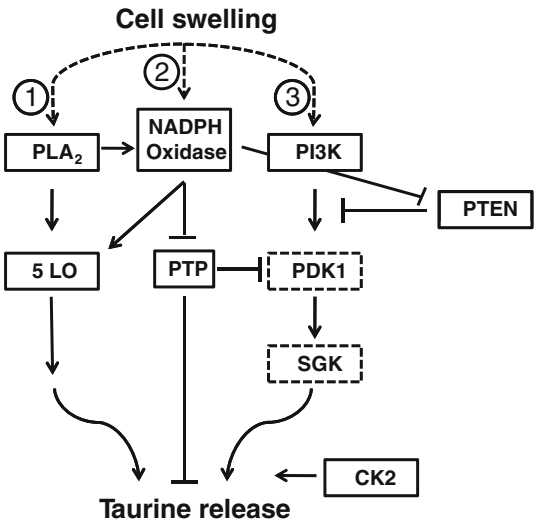

Fig. 9 Signalling cascades involved in swelling-induced taurine release. The model is described in the text. Conversion of an increased cell volume to increased PLA_2, NADPH oxidase and PI3K activity (*dashed lines*) is unknown. The PLA_2—5-LO cascade (*1*) is described in (Hoffmann et al. 2009; Lambert 2004; Lambert and Pedersen 2006; Pedersen et al. 2006), the role of NADPH oxidase-ROS-PTP (*2*) is described in (Lambert 2007; Friis et al. 2008; Lambert 2003; Ortenblad et al. 2003), activation of PI3K (*3*) is found in (Wang et al. 2004; Nielsen et al. 2008) and the downstream PDK1-SGK cascade is described in (Wang et al. 2004). Regulation of PDK1 by PTP is described in (Prasad et al. 2000). CK2 seems to modulate swelling-induced taurine efflux downstream to (*1*), (*2*) and (*3*)

phosphorylation sites and whether CK2 modulates channel activity directly.

CK2: a putative manager of volume regulatory systems and cell fate

CK2 expression is upregulated in several cancer types, including ELA cells, as a result of dysfunctional regulation caused by the already high proliferative state (Guerra and Issinger 1999; Trembley et al. 2009; Ahmad et al. 2008). Down-regulation of CK2 by RNA interference results in inhibition of cell-cycle progression, as well as sensitization towards apoptosis (Guerra and Issinger 1999; St-Denis and Litchfield 2009). CK2 is, in addition to being vital to cell cycle, a potent suppressor of apoptosis as the CK2 phosphorylation sequence often is a part of the caspase consensus sequence (Ortenblad et al. 2003). Cancer-treatment by targeting CK2 is now under investigation (see (Zhu et al. 2009; Ruzzene and Pinna 2010)). Apoptosis is accompanied by net loss of organic osmolytes in parallel to loss of potassium and chloride, and inhibition of the volume-sensitive ion channels impairs the apoptotic response (Poulsen et al. 2010). Our data indicate that inhibition of CK2 results in accelerated RVD-response even though the open-probability of the taurine efflux pathways is reduced. Thus, inhibition of CK2 as a part of cancer-treatment will promote reduction in cell volume and hence the apoptotic process. Whether the effect on the taurine transport systems, i.e. reduced open-probability of the release systems and increased taurine uptake by TauT, will affect the apoptotic process is currently under investigation.

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References

Ahmad KA, Wang G, Unger G, Slaton J, Ahmed K (2008) Protein kinase CK2—a key suppressor of apoptosis. Adv Enzyme Regul 48:179–187

Allen D, Fakler B, Maylie J, Adelman JP (2007) Organization and regulation of small conductance Ca2+ -activated K+ channel multiprotein complexes. J Neurosci 27(9):2369–2376

Arrigoni G, Marin O, Pagano MA, Settimo L, Paolin B, Meggio F et al (2004) Phosphorylation of calmodulin fragments by protein kinase CK2. Mechanistic aspects and structural consequences. Biochemistry 43(40):12788–12798

Bibby AC, Litchfield DW (2005) The multiple personalities of the regulatory subunit of protein kinase CK2: CK2 dependent and CK2 independent roles reveal a secret identity for CK2beta. Int J Biol Sci 1(2):67–79

Bildl W, Strassmaier T, Thurm H, Andersen J, Eble S, Oliver D et al (2004) Protein kinase CK2 is coassembled with small conductance Ca2+ -activated K+ channels and regulates channel gating. Neuron 43(6):847–858

Bunney TB, Katan M (2010) Phosphoinositide signaling in cancer: beyond PI3K and PTEN. Nat Rev Cancer 10(5):342–352

Diaz-Elizondo J, Chiong M, Rojas-Rivera D, Olea-Azar C, Kwon HM, Lavandero S (2006) Reactive oxygen species inhibit hyposmotic stress-dependent volume regulation in cultured rat cardiomyocytes. Biochem Biophys Res Commun 350(4):1076–1081

Filhol O, Cochet C (2009) Cellular functions of Protein kinase CK2: a dynamic affair. Cell Mol Life Sci 66(11–12):1830–1839

Fiol CJ, Mahrenholz AM, Wang Y, Roeske RW, Roach PJ (1987) Formation of protein kinase recognition sites by covalent modification of the substrate. Molecular mechanism for the synergistic action of casein kinase II and glycogen synthase kinase 3. J Biol Chem 262(29):14042–14048

Frame S, Cohen P (2001) GSK3 takes centre stage more than 20 years after its discovery. Biochem J 359(Pt 1):1–16

Friis MB, Vorum KG, Lambert IH (2008) Volume-sensitive NADPH oxidase activity and taurine efflux in NIH3T3 mouse fibroblasts. Am J Physiol Cell Physiol 294(6):C1552–C1565

Guerra B, Issinger OG (1999) Protein kinase CK2 and its role in cellular proliferation, development and pathology. Electrophoresis 20(2):391–408

Hoffmann EK, Lambert IH, Pedersen SF (2009) Physiology of cell volume regulation in vertebrates. Physiol Rev 89(1):193–277

Huyer G, Liu S, Kelly J, Moffat J, Payette P, Kennedy B et al (1997) Mechanism of inhibition of protein-tyrosine phosphatases by vanadate and pervanadate. J Biol Chem 272(2):843–851

Jacobsen JG, Smith LH (1968) Biochemistry and physiology of taurine and taurine derivatives. Physiol Rev 48(2):424–511

Jacobsen JH, Clement CA, Friis MB, Lambert IH (2008) Casein kinase 2 regulates the active uptake of the organic osmolyte taurine in NIH3T3 mouse fibroblasts. Pflugers Arch 457(2):327–337

Jenkins CM, Wolf MJ, Mancuso DJ, Gross RW (2001) Identification of the calmodulin-binding domain of recombinant calcium-independent phospholipase A2beta implications for structure and function. J Biol Chem 276(10):7129–7135

Lai JP, Dalton JT, Knoell DL (2007) Phosphatase and tensin homologue deleted on chromosome ten (PTEN) as a molecular target in lung epithelial wound repair. Br J Pharmacol 152(8): 1172–1184

Lambert IH (1998) Regulation of the taurine content in Ehrlich ascites tumour cells. Adv Exp Med Biol 442:269–276

Lambert IH (2003) Reactive oxygen species regulate swelling-induced taurine efflux in NIH3T3 mouse fibroblasts. J Membr Biol 192(1):19–32

Lambert IH (2004) Regulation of the cellular content of the organic osmolyte taurine in mammalian cells. Neurochem Res 29(1):27–63

Lambert IH (2007) Activation and inactivation of the volume-sensitive taurine leak pathway in NIH3T3 fibroblasts and ehrlich lettre ascites cells. Am J Physiol Cell Physiol 293(1):C390–C400

Lambert IH, Hoffmann EK (1993) Regulation of taurine transport in Ehrlich ascites tumor cells. J Membr Biol 131(1):67–79

Lambert IH, Pedersen SF (2006) Multiple PLA2 isoforms regulate taurine release in NIH3T3 mouse fibroblasts. Adv Exp Med Biol 583:99–108

Lambert IH, Sepulveda FV (2000) Swelling-induced taurine efflux from HeLa cells: cell volume regulation. Adv Exp Med Biol 483:487–495

Lambert IH, Nielsen JH, Andersen HJ, Ortenblad N (2001) Cellular model for induction of drip loss in meat. J Agric Food Chem 49(10):4876–4883

Lambert IH, Pedersen SF, Poulsen KA (2006) Activation of PLA2 isoforms by cell swelling and ischaemia/hypoxia. Acta Physiol (Oxf) 187(1–2):75–85

Lambert IH, Hoffmann EK, Pedersen SF (2008) Cell volume regulation: physiology and pathophysiology. Acta Physiol Scand 194:255–282

Lang F (2007) Mechanisms and significance of cell volume regulation. J Am Coll Nutr 26(5):613S–623S

Lang F, Strutz-Seebohm N, Seebohm G, Lang UE (2010) Significance of SGK1 in the regulation of neuronal function. J Physiol

Lee SR, Kwon KS, Kim SR, Rhee SG (1998) Reversible inactivation of protein-tyrosine phosphatase 1B in A431 cells stimulated with epidermal growth factor. J Biol Chem 273(25):15366–15372

Lourenço R, Camilo ME (2002) Taurine: a conditionally essential amino acid in humans? An overview in health and disease. Nutrición Hospitalaria 17(6):262–270

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the folin phenol reagent. J Biol Chem 193(1):265–275

Mancuso DJ, Jenkins CM, Gross RW (2000) The genomic organization, complete mRNA sequence, cloning, and expression of a novel human intracellular membrane-associated calcium-independent phospholipase A(2). J Biol Chem 275(14):9937–9945

Meggio F, Pinna LA (2003) One-thousand-and-one substrates of protein kinase CK2? Faseb J 17(3):349–368

Meng TC, Fukada T, Tonks NK (2002) Reversible oxidation and inactivation of protein tyrosine phosphatases in vivo. Mol Cell 9(2):387–399

Moran J, Miranda D, Pena-Segura C, Pasantes-Morales H (1997) Volume regulation in NIH/3T3 cells not expressing P-glycoprotein. II. Chloride and amino acid fluxes. Am J Physiol 272(6 Pt 1):C1804–C1809

Nielsen MB, Christensen ST, Hoffmann EK (2008) Effects of osmotic stress on the activity of MAPKs and PDGFR-beta-mediated signal transduction in NIH-3T3 fibroblasts. Am J Physiol Cell Physiol 294(4):C1046–C1055

Ortenblad N, Young JF, Oksbjerg N, Nielsen JH, Lambert IH (2003) Reactive oxygen species are important mediators of taurine release from skeletal muscle cells. Am J Physiol Cell Physiol 284(6):C1362–C1373

Pagano MA, Poletto G, Di MG, Cozza G, Ruzzene M, Sarno S et al (2007) Tetrabromocinnamic acid (TBCA) and related compounds represent a new class of specific protein kinase CK2 inhibitors. Chembiochem 8(1):129–139

Pagano MA, Bain J, Kazimierczuk Z, Sarno S, Ruzzene M, Di MG et al (2008) The selectivity of inhibitors of protein kinase CK2: an update. Biochem J 415(3):353–365

Park HS, Lee SM, Lee JH, Kim YS, Bae YS, Park JW (2001) Phosphorylation of the leucocyte NADPH oxidase subunit p47(phox) by casein kinase 2: conformation-dependent phosphorylation and modulation of oxidase activity. Biochem J 358:783–790

Pedersen SF, Poulsen KA, Lambert IH (2006) Roles of phospholipase A2 isoforms in swelling- and melittin-induced arachidonic acid release and taurine efflux in NIH3T3 fibroblasts. Am J Physiol Cell Physiol 291(6):C1286–C1296

Poulsen KA, Litman T, Eriksen J, Mollerup J, Lambert IH (2002) Downregulation of taurine uptake in multidrug resistant Ehrlich ascites tumor cells. Amino Acids 22(4):333–350

Poulsen KA, Andersen EC, Hansen CF, Klausen TK, Hougaard C, Lambert IH et al (2010) Deregulation of apoptotic volume decrease and ionic movements in multidrug-resistant tumor cells: role of chloride channels. Am J Physiol Cell Physiol 298(1):C14–C25

Prasad N, Topping RS, Zhou D, Decker SJ (2000) Oxidative stress and vanadate induce tyrosine phosphorylation of phosphoinositide-dependent kinase 1 (PDK1). Biochemistry 39(23): 6929–6935

Rao RK, Clayton LW (2002) Regulation of protein phosphatase 2A by hydrogen peroxide and glutathionylation. Biochem Biophys Res Commun 293(1):610–616

Ruzzene M, Pinna LA (2010) Addiction to protein kinase CK2: a common denominator of diverse cancer cells? Biochim Biophys Acta 1804(3):499–504

Sarkadi B, Attisano L, Grinstein S, Buchwald M, Rothstein A (1984) Volume regulation of Chinese hamster ovary cells in anisoosmotic media. Biochim Biophys Acta 774(2):159–168

Sarno S, Pinna LA (2008) Protein kinase CK2 as a druggable target. Mol Biosyst 4(9):889–894

Schmid AC, Byrne RD, Vilar R, Woscholski R (2004) Bisperoxovanadium compounds are potent PTEN inhibitors. FEBS Lett 566(1–3):35–38

Shimoyama Y, Sakamoto R, Akaboshi T, Tanaka M, Ohtsuki K (2001) Characterization of secretory type IIA phospholipase A2 (sPLA2-IIA) as a glycyrrhizin (GL)-binding protein and the GL-induced inhibition of the CK-II-mediated stimulation of sPLA2-IIA activity in vitro. Biol Pharm Bull 24(9):1004–1008

Silva A, Yunes JA, Cardoso BA, Martins LR, Jotta PY, Abecasis M et al (2008) PTEN posttranslational inactivation and hyperactivation of the PI3K/Akt pathway sustain primary T cell leukemia viability. J Clin Invest 118(11):3762–3774

St-Denis NA, Litchfield DW (2009) From birth to death: the role of protein kinase CK2 in the regulation of cell proliferation and survival. Cell Mol Life Sci 66(11–12):1817–1829

Thornburg W, Lindell TJ (1977) Purification of rat liver nuclear protein kinase NII. J Biol Chem 252(19):6660–6665

Trembley JH, Wang G, Unger G, Slaton J, Ahmed K (2009) CK2: a key player in cancer biology. Cell Mol Life Sci 66(11–12): 1858–1867

van Diepen MT, Parsons M, Downes CP, Leslie NR, Hindges R, Eickholt BJ (2009) MyosinV controls PTEN function and neuronal cell size. Nat Cell Biol 11(10):1191–1196

Vanhaesebroeck B, Leevers SJ, Panayotou G, Waterfield MD (1997) Phosphoinositide 3-kinases: a conserved family of signal transducers. Trends Biochem Sci 22(7):267–272

Villumsen KR, Duelund L, Lambert IH (2010) Acute cholesterol depletion leads to net loss of the organic osmolyte taurine in Ehrlich Lettre tumor cells. Amino Acids. doi:10.1007/s00726-010-0621-4

Voss JW, Pedersen SF, Christensen ST, Lambert IH (2004a) Regulation of the expression and subcellular localisation of the taurine transporter TauT in mouse NIH3T3 fibroblasts. Eur J Biochem 271:4646–4658

Voss JW, Pedersen SF, Christensen ST, Lambert IH (2004b) Regulation of the expression and subcellular localization of the taurine transporter TauT in mouse NIH3T3 fibroblasts. Eur J Biochem 271(23–24):4646–4658

Wang GX, McCrudden C, Dai YP, Horowitz B, Hume JR, Yamboliev IA (2004) Hypotonic activation of volume-sensitive outwardly rectifying chloride channels in cultured PASMCs is modulated by SGK. Am J Physiol Heart Circ Physiol 287(2):H533–H544

Weston CR, Davis RJ (2001) Signal transduction: signaling specificity—a complex affair. Science 292(5526):2439–2440

Zhu D, Hensel J, Hilgraf R, Abbasian M, Pornillos O, Deyanat-Yazdi G et al (2009) Inhibition of protein kinase CK2 expression and activity blocks tumor cell growth. Mol Cell Biochem