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Lysophosphatidylcholine Induces Taurine Release from HeLa Cells

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Abstract. The putative role of lysophospholipids in activation and regulation of the volume-sensitive taurine efflux was investigated in HeLa cells using tracer technique. Lysophosphatidylcholine (LPC, 10 μM) with oleic acid increased taurine efflux during hypotonic and isotonic conditions. Substituting palmitic or stearic acid for oleic acid enhanced taurine release during isotonic conditions, whereas ethanolamine, serine or inositol containing lysophospholipids were ineffective. High concentrations of LPC (25 μM) induced Ca^{2+} influx, loss of adenosine nucleotides, taurine and the Ca^{2+}-sensitive probe Fura-2, and thus reflected a general breakdown of the membrane permeability barrier. Low concentrations of LPC (5–10 μM) solely induced taurine efflux. The LPC-induced taurine release was unaffected by anion channel blockers (DIDS, MK196) and the 5-lipoxygenase inhibitor ETH 615-139, which all blocked the volume sensitive taurine efflux. Furthermore, LPC-induced taurine release was reduced by antioxidants (NDGA, vitamin E) and the protein tyrosine kinase inhibitor genistein. The swelling-induced taurine efflux was in the absence of LPC unaffected by vitamin E, blocked by genistein, and increased by H_2O_2 and the protein tyrosine phosphatase inhibitor vanadate. It is suggested that low concentrations of LPC permeabilizes the plasma membrane in a Ca^{2+}-independent process that involves generation of reactive oxygen species and tyrosine phosphorylation, and that LPC is not a second messenger in activation of the volume sensitive taurine efflux in HeLa cells.

Key words: Cell volume regulation — Membrane permeabilization — Vitamin E — Tyrosine phosphorylation — Lysophospholipids

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Introduction

Taurine, 2-aminoethanesulfonic acid is an important organic osmolyte in mammalian cells (*see* Huxtable, 1992). Taurine is accumulated via a Na^+- and in some cases Cl^--dependent process (*see* Huxtable, 1992) and released following hypotonic exposure often concurrently with K^+ and Cl^- (*see* Lambert, 1998; Hoffmann & Mills, 1999). The identity of the swelling-induced taurine efflux pathway is not known. Taurine and Cl^- apparently share the volume-sensitive organic anion channel (VSOAC) in Madin Darby kidney cells (Banderali & Roy, 1992) and C6 glioma cells (Jackson & Strange, 1993), whereas they leave the Ehrlich ascites tumor cells (Lambert & Hoffmann, 1994; Lambert, 1998) and the HeLa cells (Stutzin et al., 1999) via separate, volume-sensitive pathways.

Hydrolysis of membrane phospholipids to lysophospholipids and free fatty acids by phospholipase A_2 (PLA_2) has turned out to be an important, initial step in the swelling-induced release of osmolytes in Ehrlich tumor cells (Lambert & Hoffmann, 1991), human blood platelets (Margalit et al., 1993*a*), mudpuppy red blood cells (Light et al., 1997), CHP-100 neuroblastoma cells (Basavappa et al., 1998), cerebellar granule neurons (Morales-Mulia et al., 2000) and HeLa cells (Lambert & Sepúlveda, 2000). Several forms of PLA_2 have been identified in mammalian cells and they differ with respect to Ca^{2+} dependence, molecular size and substrate preference (*see* Balsinde et al., 1999; Gijón & Leslie, 1999). In the case of the Ehrlich cells (Thoroed et al., 1997), CHP-100 neuroblastoma cells (Basavappa et al., 1998) and cerebellar granule neurons (Morales-Mulia et al., 2000) it has been demonstrated that the volume-sensitive PLA_2 is the Ca^{2+}-dependent, 85 kDa, cytosolic PLA_2 ($cPLA_2$), which preferentially hydrolyses phospholipids containing arachidonic acid at the *sn*-2 position (*see* Balsinde et al., 1999; Gijón & Leslie, 1999) leaving behind lysophospholipids, predominantly lysophosphati-

dylcholine (LPC). $cPLA_2$ is present in HeLa cells and it has recently been demonstrated that it undergoes phosphorylation as well as translocation from cytosol to membranes when the HeLa cells attach and spread on a gelatin matrix (Crawford & Jacobson, 1998). Translocation of $cPLA_2$ to membranes is observed in response to (i) an increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$) in a process that involves a Ca^{2+} binding domain within the enzyme (*see* Balsinde et al., 1999; Wong et al., 1998), (ii) following cell swelling in Ehrlich cells (Pedersen et al., 2000), and (iii) application of LPC in cardiac myoblastic H9c2 cells (Golfman et al., 1999). In the case of Ehrlich cells it appears that it is the isoform $cPLA_{2\alpha}$ that translocates to the nuclear envelope and form hot spotlike clusters following an increase in $[Ca^{2+}]_i$ or osmotic cell swelling, whereas the isoform $cPLA_{2\gamma}$ does not translocate during the same conditions (Pedersen et al., 2000). The PLA_2, activated by cell swelling in HeLa cells, has not been identified.

Arachidonic acid, released by PLA_2, serves as a precursor for biologically active eicosanoids among which leukotrienes and hepoxilins have been ascribed roles as potential second messengers in the swelling-induced activation of the organic/inorganic osmolyte transporting systems in Ehrlich cells (Lambert et al., 1987) and human blood platelets (Margalit et al., 1993*b*), respectively. On the other hand, little attention has been drawn to the putative role of the lysophospholipids in the activation and regulation of the cellular osmolyte content. An increased level of LPC is observed in, e.g., Ehrlich cells after cell swelling (Thoroed et al., 1997), snake venom-treated phosphatidylcholine suspensions (*see* Weltzien, 1979), atherosclerotic tissue (Portman & Alexander, 1969), ischemic heart (Kinnaird, Choy & Man, 1988) and in oxidized lipoproteins (Liu et al., 1994). In the case of ischemic myocardium it has been estimated that during in vivo conditions the free concentration of LPC may rise to 20 μM (Shaikh & Downar, 1981; Yu et al., 1998). LPC is known to increase $[Ca^{2+}]_i$ in cardiomyocytes (Chen et al., 1997; Yu et al., 1998), human endothelial cells (Wong et al., 1998) and cardiac myoblastic H9c2 cells (Golfman et al., 1999), an effect which in itself accelerates loss of osmolytes and cell water following osmotic cell swelling (Jørgensen et al., 1997). LPC also increases $cPLA_2$ activity in human endothelial cells (Wong et al., 1998) and rat cardiomyocytes (Chen et al., 1997), resulting in an enhanced availability of arachidonic acid and lysophospholipids (positive feedback). More recently it has been demonstrated that LPC generates superoxide anions in isolated neutrophils and that the LPC-induced signalling pathway involves the phosphatidyl inositol 3-kinase (Nishioka et al., 1998). However, although LPC is a normal constituent in plasma, lipoproteins and almost every biological membrane an excessive incorporation of this amphiphilic lysophospholipid in the plasma membrane will cause breakdown of the membrane permeability barrier (Weltzien, 1979; Lundbæk & Andersen, 1994).

As PLA_2 activity is required for activation of the volume-sensitive taurine efflux pathway in HeLa cells (Lambert & Sepúlveda, 2000), we have characterized the LPC-induced activation of taurine efflux in HeLa cells in order to elucidate whether a putative increase in LPC is involved in the swelling-induced release of taurine.

Materials and Methods

Cell Culture

The human cervical carcinoma cell line, HeLa, was maintained as a monolayer culture in Eagle's minimum essential medium containing 10% v/v newborn calf serum, 2 mM glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. Incubation temperature was 37°C and CO_2 5%. The cell culture was split (1:16) twice a week using 0.25% trypsin in PBS to detach the cells.

Inorganic Media

The PBS contained in mM: 137 NaCl, 2.6 KCl, 6.5 Na_2HPO_4, and 1.5 KH_2PO_4. Isosmotic NaCl contained in mM: 143 NaCl, 5 KCl, 1 Na_2HPO_4, 1 $CaCl_2$, 0.1 $MgSO_4$, 5 glucose, and 10 *N*-2-hydroxyethyl piperazine-*N*′-2-ethanesulfonic acid (HEPES). Isosmotic KCl solution contained in mM: 150 KCl, 1.3 $CaCl_2$, 0.5 $MgCl_2$, and 10 HEPES. Hyposmotic KCl solution was obtained by reduction of the KCl in the isosmotic KCl solution to 95 mM, with the other components remaining unchanged. pH was in all solutions adjusted at 7.4. The osmolarity of the isotonic and the hypotonic solution were estimated at 295 and 195 mosmole per liter, respectively (Knaur, Halbmikro-Osmometer). Solutions containing high concentrations of K^+ were used in order to eliminate the outward transmembrane K^+ gradient, i.e., the driving force for RVD and thereby prolonging the volume-activated response in hyposmotic media (*see* Kirk & Kirk, 1993).

Chemicals

Penicillin, streptomycin, glutamine, newborn calf serum, modified Eagle's medium and trypsin were from Life Technologies (Denmark). [^{14}C]-taurine was from NEN Life Science Products. MK196 was a gift from Merck Shape and Dohme (Denmark). ETH 615-139 was donated by Dr. I. Ahnfelt-Rønne (Løvens Kemiske Fabrik, Denmark). All other compounds were from Sigma Chemical.

Efflux Measurements—Estimation of Rate Constants

Taurine efflux from HeLa cells was measured at room temperature (~20°C) as described previously by Hall and coworkers (1996). Cells were grown to 80% confluence in 35-mm diameter polyethylene dishes and loaded with [^{14}C]-taurine by preincubating the cells for 2 hr at 37°C with 0.14 mCi/ml [^{14}C]-taurine in either serum-free NaCl medium containing 5 mM glucose (serum starvation) or Eagle's medium containing 10% v/v newborn calf serum. Prior to the efflux experiments the incubation solution was removed and the cells washed 5

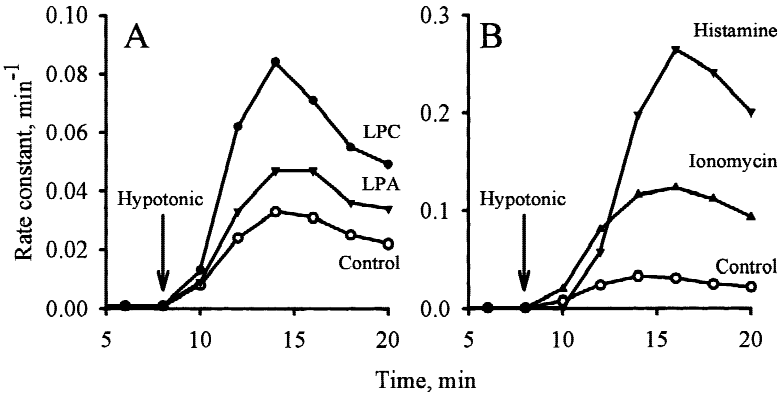

Fig. 1. Effect of lysophospholipids, ionomycin and histamine on the swelling-induced taurine efflux from HeLa cells. Cells, grown to 80% confluence, were serum starved and preloaded with [^{14}C]-taurine for 2 hr in NaCl medium containing 5 mM glucose. The efflux experiments were performed in KCl media and the rate constant estimated as described in Materials and Methods. The osmolarity was reduced to 2/3 of the isotonic value at the time indicated by the arrow. (*A*) 10 μM LPA or 10 μM LPC, both with oleic acid in the *sn*-1 position, were added at the time of hypotonic exposure. (*B*) 250 nM ionomycin or 10 μM histamine were added at the time of hypotonic exposure. The number of independent sets of experiments (*n*) is indicated in Table 1.

times by gentle addition and removal of 1 ml isosmotic KCl solution in order to remove excess [^{14}C]-taurine. The solution was removed and discarded after the final wash and another 1 ml of isosmotic solution was added to the dish. At 2 min time intervals the solution was replaced by another 1 ml aliquot. The KCl solution removed from the dish was transferred to a scintillation vial for estimation of ^{14}C activity (β-scintillation counting, Ultima Gold™). This procedure was repeated for 20–30 min. The amount of [^{14}C]-taurine remaining inside the cells at the end of the efflux experiment was estimated by treating the cells with 1 ml 0.5 N NaOH for 2 hr, washing the dishes 2 times with distilled water and estimating the ^{14}C activity in the NaOH as well as in water washouts. The natural logarithm to the fraction of ^{14}C activity remaining in the cells at a given time (*t*) was plotted *vs.* time and the rate constant for the taurine efflux was estimated as the negative slope of the graph between time point *t* and its proceeding time point.

Estimation of the ATP, ADP and AMP by Reversed Phase Ion Pair HPLC

Cells, grown in 83 cm^2 flasks, were washed 5 times with isosmotic KCl medium. The solution was removed, a further 5 ml experimental solution added and the cells incubated for 20 min at room temperature. The procedures used for extraction and detection of the ATP, ADP and AMP content are described by Kramhøft, Mollerup and Lambert (1997) and Murray, Thomson and McGill (1985), respectively. Briefly, the solution covering the cells was removed, filtered (0.22 μm Millex-GV, Millipore) and used for estimation of nucleotides in the extracellular compartment. Cells remaining in the flask were washed 3 times with ice-cold isosmotic KCl medium, the medium removed by suction and the cellular nucleotides extracted with perchloric acid (0.14 M). The acidic solution was transferred to glass tubes, neutralized by addition of 2.5 volumes of freshly prepared tri-N-octylamine (0.5 M) in freon, mixed 3 times 20 sec, left for 10 min, centrifuged (15,000 × *g*, 5 sec), filtered and used for estimation of nucleotides in the cellular compartment. Nucleotides were separated by reversed phase ion pair HPLC on a Gilson HPLC system, using a C18 Nucleosil (4 × 250 mm, 5 μm particles) column, isocratic elution (1 ml/min) with a mobile phase consisting of 1.95 mM tetrabutylammonium hydrogen sulfate (ion-pairing agent), 0.1 M KH_2PO_4/K_2HPO_4, (pH 7.0), 9.5% methanol (v/v) and detection at 254 nm. ATP, ADP and AMP standards in water were used for estimation of retention times and the total content in the cellular and the extracellular compartment.

Statistical Evaluation

Data were in all cases analyzed by a paired Students *t*-test and $P < 0.05$ was considered as statistically significant.

Results

It has recently been shown that PLA_2 activity is required for the swelling-induced activation of taurine efflux in HeLa cells (Lambert & Sepúlveda, 2000) and the experiments presented in Figs. 1 and 2 and Tables 1 and 2 were performed in order to investigate whether the lysophospholipids, generated by PLA_2, could induce or stimulate taurine efflux from HeLa cells. As lysophospholipids are natural components of serum we performed all experiments on HeLa cells that were serum deprived for 2 hr before initiation of the experiments. Figure 1*A* shows that addition of 10 μM LPC or lysophosphatidic acid (LPA), both with oleic acid, at the time of hypotonic exposure increases the swelling-induced taurine efflux from HeLa cells. It is estimated that the maximal rate constant for taurine efflux obtained after hypotonic exposure is significantly increased 2.5-, 1.5- and 2.2-fold following addition of 10 μM LPC or 10 μM/25 μM LPA, respectively (Table 1), i.e., LPC appears to be more potent than LPA. Figure 1*B* and Table 1 show that the effect of LPC and LPA on the swelling-induced taurine efflux is mimicked by addition of the Ca^{2+} ionophore ionomycin or the Ca^{2+} mobilizing agonist histamine. Both LPC (Chen et al., 1997; Yu et al., 1998; Wong et al., 1998) and LPA (*see* Moolenaar et al., 1997; Goetzl & An, 1998) have been reported to increase $[Ca^{2+}]_i$, indicating that the potentiating effect of LPC and LPA on the swelling-induced taurine efflux could be secondary to an increase in $[Ca^{2+}]_i$.

If LPC or LPA are considered as second messengers in the swelling-induced activation of the taurine efflux in HeLa cells, it would be expected that addition of these

Table 1. Potentiation of the swelling-induced taurine efflux by lysophospholipids, ionomycin and histamine

		Rate constant relative value	*n*	*P*
Control		1		
LPC	10 μM	2.5 ± 0.1	4	<0.001
LPA	10 μM	1.5 ± 0.1	7	<0.01
	25 μM	2.2 ± 0.1	6	<0.001
Ionomycin	250 nM	3.2 ± 0.5	4	<0.02
	500 nM	4.4 ± 0.4	3	<0.04
Histamine	10 μM	4.0 ± 0.3	4	<0.01

Serum starved cells were prepared and LPC (oleic acid in *sn*-1), LPA (oleic acid in *sn*-1), ionomycin or histamine were added at the time of hypotonic exposure as described in the legend to Fig. 1. The maximal rate constants following hypotonic exposure are given relative to the hypotonic control, which was estimated at 0.056 ± 0.006 min^{-1} in 15 sets of experiments. All values are mean ± SEM and *n* indicates the number of paired sets of experiments. *P* indicates the statistical value in a paired Student's *t*-test, in which experimental values were tested against the hypotonic control value.

lysophospholipids should induce taurine release from HeLa cells suspended in isotonic solutions. From Fig. 2*A* and Table 2 it is seen that addition of LPC with oleic acid indeed induce taurine release during isotonic conditions, whereas LPA has only a minor effect. Substituting a saturated fatty acid (palmitic acid/stearic acid) for the unsaturated fatty acid (oleic acid) potentiates the taurine releasing effect of the lysophospholipid (Fig. 2; Table 2). On the other hand, lysophosphatidyl serine (LPS), lysophosphatidyl inositol (LPI), lysophosphatidyl ethanolamine (LPE) or palmitic acid only produce minor or no increase in the rate constant for taurine efflux from HeLa cells suspended in isotonic solution (Table 2). Thus, LPC with palmitic acid has maximal effect on the taurine efflux and was accordingly used in the remaining part of the present study. From Fig. 2*B* and Table 2 it is seen that the maximal rate constant for the LPC-induced taurine efflux is concentration dependent, and that the onset of taurine release is delayed by 2–4 min following addition of 5 and 10 μM LPC but almost immediate following addition of 25 μM LPC. In Fig. 2 it is also seen that the effect of LPC on taurine release is transient which could indicate that HeLa cells become depleted for [^{14}C]-labeled taurine during the efflux experiment (*see* Materials and Methods). However, in 3 sets of experiments it was estimated that at the end of the efflux experiment, i.e., after 22 min exposure to 5 μM, 10 μM or 25 μM LPC, the HeLa cells contained 15 ± 2%, 3 ± 1% and 1% of the initial amount of tracer, respectively. Thus, at 5 μM LPC the transient nature of the LPC-induced taurine efflux does not reflect a depletion of the [^{14}C]-taurine pool but rather that HeLa cells have a capacity for reacylation of LPC by acyltransferases or cleavage of LPC by lysophospholipases or phospholipases into, e.g., glycerophosphorylcholine, monoacylglycerol or phosphatidic acid. These possibilities were not investigated in the present work. It is noted that LPC also induces a transient increase of [^{14}C]-labeled aminoisobutyric acid from HeLa cells (*data not shown*), i.e., LPC increases the permeability of HeLa cells toward sulphonic as well as carboxylic amino acids.

From a series of experiments with the Ca^{2+} sensitive, fluorescent probe Fura-2 it was observed that addition of 5 and 10 μM LPC to HeLa cells suspended in hypotonic solution in 2 out of 3 experiments produced no increase in $[Ca^{2+}]_i$ and in 1 out of 3 experiments gave a slow continuous increase in $[Ca^{2+}]_i$ (*data not shown*). As addition of 10 μM histamine increases $[Ca^{2+}]_i$ significantly within seconds during isotonic conditions (*data not shown*) with no concomitant increase in taurine release (Table 2), and addition of 10 μM LPC induces taurine release and no consistent increase in $[Ca^{2+}]_i$, it is suggested that LPC-induced taurine release in HeLa cells is Ca^{2+}-independent. On the other hand, addition of 15 μM LPC induced a substantial increase in $[Ca^{2+}]_i$ in HeLa suspended in Ca^{2+} containing media, whereas it had no effect on $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+} (*data not shown*). On a longer time schedule, i.e., more than 10 min, 15 μM LPC also induced a loss of the fluorescent Ca^{2+}-sensitive probe from the cells. In order to test whether high concentrations of LPC increased the permeability to other cellular components we examined its effect on the cellular adenosine nucleotide content. Figure 3 demonstrates the cellular to extracellular distribution of total nucleotide content, i.e., ATP, ADP plus AMP in HeLa cells exposed for 20 min to isotonic medium, hypotonic medium or isotonic medium containing either 25 μM LPC or 10 μg/ml of digitonin. Digitonin is a membrane permeabilizing agent and was added as a positive control. It is seen that during isotonic conditions 90% of the nucleotides is in the cells and that hypotonic exposure does not alter this distribution, i.e., the HeLa cells seem not to loose nucleotides following osmotic cell swelling. In contrast, both LPC and digitonin release nucleotides from the HeLa cells (Fig. 3). Addition of 5 to 10 μg/ml digitonin also leads to an increased efflux of taurine from HeLa cells suspended in isotonic medium (Table 2). A simple assumption is that exposure of HeLa cells to high concentrations of LPC, i.e., 15–25 μM, leads to a general breakdown of the membrane permeability barrier. It is emphasized that trypan blue exclusion in cells treated for 10 min with 25 μM LPC revealed that less than 2% of the cells were stained (value not different from untreated control cells), indicating that the LPC-induced changes in membrane permeability are not a consequence of a reduced cell viability.

If LPC is released during osmotic cell swelling and subsequently plays a role as a second messenger in the activation of the volume sensitive taurine efflux path-

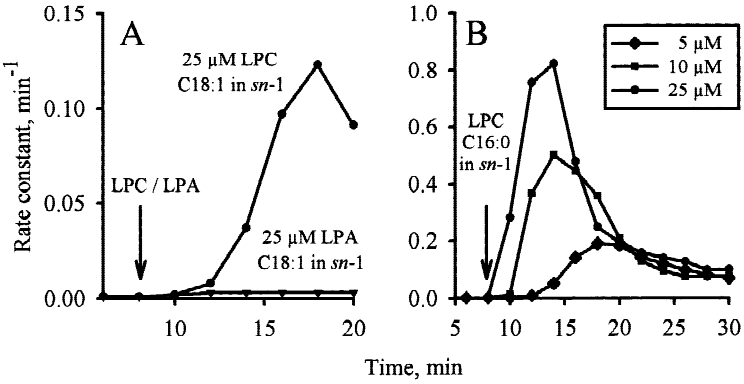

Fig. 2. Effect of lysophospholipids on taurine release from HeLa cell suspended in isotonic medium. Cells were grown to 80% confluence, serum starved and preloaded with [^{14}C]-taurine for 2 hr in NaCl medium containing 5 mM glucose. The efflux experiments were performed in isotonic KCl medium and the rate constant estimated as described in Materials and Methods. All additions were made at the time indicated by the arrow. (*A*) 25 μM LPA or 25 μM LPC, both with oleic acid (C18:1) in *sn*-1. (*B*) 5–25 μM LPC with palmitic acid (C16:0) in *sn*-1. The number of independent sets of experiments (*n*) is indicated in Table 2.

Table 2. Effect of lysophospholipids, palmitic acid, ionomycin and histamine on the rate constant for taurine efflux from HeLa cells suspended in isotonic medium

	Concentration	Rate constant min^{-1}	*n*	*P*
Control		0.0011 ± 0.0001	32	
LPA, C18:1 in *sn*-1	25 μM	0.004 ± 0.001	5	<0.05
LPC, C18:1 in *sn*-1	25 μM	0.191 ± 0.093	3	<0.09
LPC, C18:0 in *sn*-1	25 μM	0.379 ± 0.005	3	<0.001
LPC, C16:0 in *sn*-1	25 μM	0.603 ± 0.043	12	<0.0001
	10 μM	0.315 ± 0.023	16	<0.0001
	5 μM	0.173 ± 0.014	3	<0.005
LPS, C16:0 in *sn*-1	25 μM	0.012 ± 0.002	5	<0.05
LPE, C16:0 in *sn*-1	25 μM	0.003 ± 0.0004	5	<0.10
LPI, C16:0/C18:0 in *sn*-1	25 μM	0.010 ± 0.006	3	<0.15
Palmitic acid	50 μM	0.005 ± 0.0003	4	<0.05
Ionomycin	250 nM	0.001 ± 0.0001	2	
Histamine	10 μM	0.001 ± 0.0001	5	<0.15
Digitonin	5 μg/ml	0.21 ± 0.04	4	<0.0001
	10 μg/ml	0.48 ± 0.02	4	<0.0001

Serum starved cells were loaded with [^{14}C]-taurine and prepared for efflux experiments as described in Materials and Methods. Lysophospholipids (LPA, LPC, LPS, LPE and LPI), palmitic acid, histamine, ionomycin and digitonin were added and the taurine efflux followed during isotonic conditions for 30 min as indicated in Fig. 2. The short hand notation C16:0, C18:0 and C18:1 indicates palmitic acid, stearic acid and oleic acid, respectively. Rate constants in the presence of LPCs and digitonin are given as the mean ± SEM of the peak values obtained after addition of the lysophospholipid (*see* Fig. 2*A* and *B*). Rate constants in controls and in the presence of palmitic acid, ionomycin and histamine are the mean ± SEM of the plateau values (minimum 4 experimental points) obtained after addition of the compounds. In the case of LPS, LPE and LPI the taurine efflux increased linearly with time following addition of the compounds and the efflux curve did not peak or level out within the experimental period (20 min). The rate constants in the presence of LPS, LPE and LPI are accordingly estimated as the mean ± SEM of the values measured 20 min after addition of the phospholipid. *n* indicates the number of independent experiments. *P* indicates the statistical value in a paired Student's *t*-test where experimental values were tested against the isotonic control value.

way, one would expect that the swelling-induced taurine efflux and the LPC-induced taurine efflux should share common features. The swelling-induced taurine efflux is inhibited by addition of an array of more or less specific anion channel inhibitors, i.e., the stilbene 4,4′-diisothiocyanatostilbene-2,2 disulphonic acid (DIDS) and the polyunsaturated arachidonic acid as well as by inhibitors of the 5-lipoxygenase (5-LO), i.e., nordihydroguaiaretic acid (NDGA) and ETH 615-139 (Hall et al., 1996; Stutzin et al., 1997; Lambert et al., 1999: Lambert & Sepúlveda, 2000). From Fig. 4 it is seen that the swelling-induced taurine efflux (open bars) is reduced in the presence of DIDS, arachidonic acid and the Cl^- channel blocker MK196 (indacrinone), whereas the LPC-

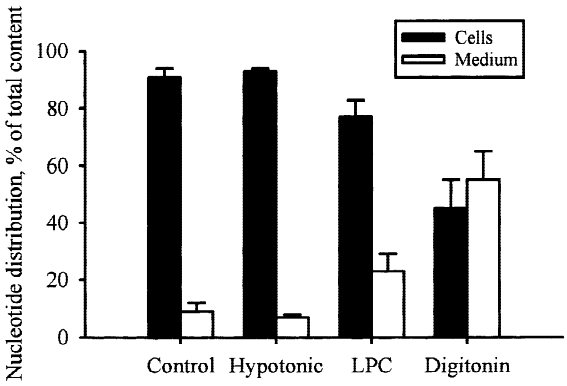

Fig. 3. Effect of hypotonic exposure and addition of LPC and digitonin on the cellular to extracellular nucleotide distribution. HeLa cells grown to 80% confluence in Eagle's medium containing 10% v/v newborn calf serum were washed in isotonic KCl medium and incubated for 20 min at room temperature in either isotonic KCl medium, hypotonic KCl medium or isotonic KCl medium containing either 25 μM LPC or 10 μg digitonin/ml. Nucleotides were extracted from the cells and the medium after 20 min and separated by reversed phase HPLC as described in Materials and Methods. The cellular and extracellular nucleotide content, i.e., the ATP, ADP plus AMP content are expressed as a percentage of the total nucleotide content (extracellular plus cellular). The values are given as mean of three independent sets of experiments ± SEM. The effect of LPC and digitonin was significant at a 0.03 and 0.02 level, respectively.

induced efflux (closed bars) is not affected by DIDS, MK196 and only partly inhibited by arachidonic acid. This is taken to indicate that the LPC-induced taurine efflux in HeLa cells differs from the swelling-induced taurine efflux pathway with respect to its sensitivity towards channel blockers. Figure 4 also demonstrates that the 5-LO inhibitor NDGA blocks the swelling-induced as well as the LPC-induced taurine efflux in HeLa cells, whereas the other 5-LO inhibitor ETH 615-139 inhibits the volume sensitive taurine efflux but stimulates the LPC-induced taurine efflux. The opposing effect of NDGA and ETH 615-139 is confirmed in Fig. 5*A*, where LPC was added during hypotonic conditions after suppression of the swelling-induced taurine efflux by the 5-LO inhibitors. ETH 615-139 and NDGA both inhibit the synthesis of leukotrienes, however ETH 615-139 is in contrast to NDGA not an anti-oxidant type of inhibitor (Kirstein, Thomsen & Ahnfelt-Rønne, 1991). From Fig. 5*B* it is seen that addition of vitamin E has no effect on the swelling-induced taurine efflux but blocks the LPC-induced taurine efflux. Addition of soybean oil, which served as vehicle for vitamin E, had no effect on the taurine efflux (*data not shown*). Thus, the effect of NDGA on the swelling-induced taurine efflux in HeLa cells reflects an inhibition of the 5-LO and not an anti-oxidant effect.

Figure 6 demonstrates that the taurine efflux induced by addition of 10 μM LPC to HeLa cells in isotonic medium (panel *A*) or by osmotic cell swelling (panel *B*) is reduced in the presence of the tyrosine kinase inhibitor genistein. Figure 6*B* also demonstrates that the swelling-induced taurine efflux is significantly stimulated by addition of the protein tyrosine phosphatase inhibitor vanadate. Vanadate had no effect on taurine release when added during isotonic conditions (*data not shown*). Thus, LPC-induced activation of taurine efflux seems to involve generation of reactive oxygen species (Fig. 5) as well as activation of a protein tyrosine kinase (Fig. 6*A*), whereas swelling-induced activation of taurine efflux involves activation of a protein tyrosine kinase/inhibition of a protein tyrosine phosphatase (Fig. 6*B*) but most probably not generation of reactive oxygen species. Protein tyrosine phosphatases has been reported to be inhibited also by H_2O_2 (*see* Cunnick et al., 1998) and in Fig. 7*A* it is seen that addition of 2 mM H_2O_2 has no effect on taurine efflux during isotonic conditions, whereas the swelling-induced taurine efflux is stimulated. In 8 sets of paired experiments it was estimated that the maximal rate constant for taurine efflux obtained after osmotic swelling in the presence of 2 mM H_2O_2 was increased to 156 ± 11% ($P < 0.001$) compared to the hypotonic control value. DIDS blocks the effect of vanadate (Fig. 6*B*) as well as the effect of H_2O_2 (Fig. 7*B*) on the swelling-induced taurine efflux, indicating that it is the DIDS and volume sensitive taurine pathway that is stimulated by vanadate and H_2O_2. H_2O_2 is required for 5-LO activity in mammalian cells (*see* Musser & Kreft, 1992) and as addition of the 5-LO inhibitor ETH 615-139 inhibits the swelling-induced taurine efflux even in the presence of H_2O_2 (Fig. 7*B*), it is assumed that the potentiating effect of H_2O_2 on the swelling-induced taurine efflux (Fig. 7*A*) reflects stimulation of the 5-LO.

Discussion

Osmotically swollen mammalian cells release ions, organic osmolytes, such as taurine, and cell water in order to restore their original cell volume (*see* Hoffmann & Mills, 1999). It has been demonstrated that activation of the volume regulatory mechanism in Ehrlich cells (*see* Thoroed et al., 1997), CHP-100 neuroblastoma cells (Basavappa et al., 1998) and cerebellar granule neurons (Morales-Mulia et al., 2000) involves the 85 kDa, $cPLA_2$. $cPLA_2$ also plays a role in receptor-mediated arachidonic acid release (Gijón & Leslie, 1999; Balsinde et al., 1999), which emphasizes that signal transduction mechanisms, normally activated by hormones, neurotransmitters and autocrine factors, also respond to changes in cell volume (*see* Lang et al., 1998). PLA_2 incorporated in liposomes has been demonstrated to be activated by osmotic swelling of the liposomes (Lehtonen & Kinnunen, 1995), indicating that changes in the lipid bilayer can act as a mechanosignal (*see* Hoffmann & Mills, 1999). PLA_2 activity is also required for

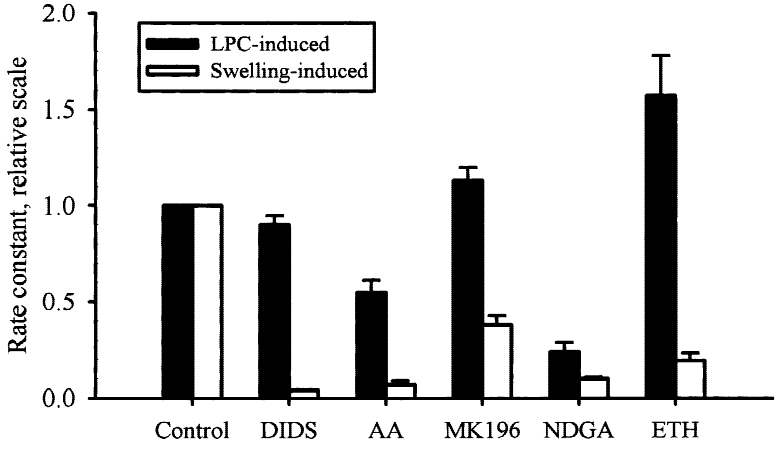

Fig. 4. Effect of channel blockers and 5-lipoxygenase inhibitors on the swelling-induced and the LPC-induced taurine efflux. Cells were grown to 80% confluence, serum starved and preloaded with [^{14}C]-taurine for 2 hr in NaCl medium containing 5 mM glucose. The efflux experiments were performed either in isotonic KCl with a shift to hypotonic KCl medium after 6–8 min (open bars) or in isotonic KCl medium with addition of 10 μM LPC after 6–8 min (closed bars) as demonstrated in Figs. 1 and 2, respectively. The total experimental time was 20–30 min. DIDS (10 μM), arachidonic acid (50 μM), MK196 (100 μM), NDGA (50 μM), and ETH 615-139 (10 μM) were present in the efflux media throughout the efflux experiment. The maximal rate constants after hypotonic exposure or after addition of LPC were estimated in the absence (control) and the presence of inhibitors. Values are given relative to the control value ± SEM, which were 0.065 ± 0.007 min^{-1} for swelling-induced and 0.39 ± 0.05 min^{-1} for LPC-induced taurine efflux. The number of experiments with hypotonic exposure//LPC was 4//4 (DIDS), 4//4 (arachidonic acid), 5//3 (MK196), 4//3 (NDGA) and 3//2 (ETH 615-139). The inhibition of the drugs on the swelling-induced taurine efflux was significant at a 0.01 level, whereas their effect on the LPC-induced taurine efflux was only significant at a 0.02 level in the case of NDGA and arachidonic acid. The stimulation of ETH 615-139 on LPC-induced efflux was significant at a 0.02 level.

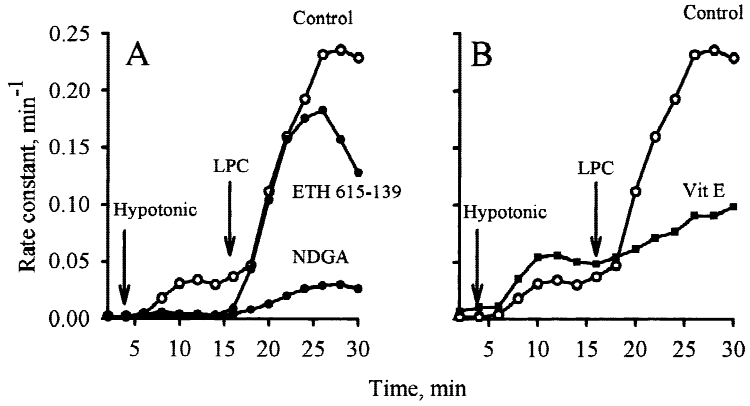

Fig. 5. Effect of 5-lipoxygenase inhibitors and Vitamin E on the swelling-induced and the LPC-induced taurine efflux. Cells grown to 80% confluence were serum starved and preloaded with [^{14}C]-taurine for 2 hr in NaCl medium containing 5 mM glucose. The efflux experiments were performed in KCl medium with a reduction in the osmolarity to 2/3 of the isotonic value at time 4 min as indicated by the arrow. NDGA (50 μM, *A*), ETH 615-139 (10 μM, *A*) and Vitamin E (20 μg/ml, *B*) were present in the efflux media throughout the efflux experiment. 10 μM LPC, with palmitic acid in *sn*-1, was added at time 16 min and the rate constant estimated as described in Materials and Methods. The curves are representative of 5 sets of identical experiments.

swelling-induced activation of taurine releasing systems in HeLa cells (Lambert & Sepúlveda, 2000) but whether the PLA_2 in question in HeLa cells is the $cPLA_{2\alpha}$ isoform, as demonstrated in Ehrlich cells (Pedersen et al., 2000), has not been established.

$cPLA_2$ cleaves the phospholipids at the *sn*-2 position giving rise to arachidonic acid as well as lysophospholipids. Arachidonic acid once released is either reacylated in the membrane or converted via the cyclooxygenase (COX1; COX2), lipoxygenase (5-LO, 12-LO or 15-LO) or cytochrome P-450 pathway. Oxidation of arachidonic acid via the 5-LO is required for activation of taurine efflux after osmotic exposure in Ehrlich cells (Lambert & Hoffmann, 1993), human fibroblasts (Mastrocola et al., 1993), fish erythrocytes (Thoroed & Fugelli, 1994), cerebellar astrocytes (Sánches-Olea et al., 1995), mudpuppy red blood cells (Light et al., 1997) as well as in HeLa cells (*see* Lambert & Sepúlveda, 2000). Furthermore, it has turned out that the 5-LO product leukotriene D_4 is responsible for the activation of the volume-sensitive taurine efflux (*see* Lambert, 1998) and the volume-sensitive K^+ efflux (Hoffmann, 1999) in Ehrlich cells, whereas the 12-LO product hepoxilin A_3 accounts for the activation of the volume regulatory response in the human platelets (Margalit et al., 1993*b*). No lipoxygenase product have yet been assigned a role as a taurine releasing messenger in HeLa cells (*see* Lambert & Sepúlveda, 2000).

Phosphatidylcholine (PC) is the main phospholipid component in eukaryotic cells and the derivative LPC mediates its effects on membrane transport either directly via a modification of the membrane permeability or indirectly via uncoupling of receptor/G-protein mediated signalling, regulation of protein kinase C (*see* Prokazova, Zvezdina & Korotaeva, 1998), generation of superoxide anions (Nishioka et al., 1998), $cPLA_2$-mediated release of arachidonic acid (Wong et al., 1998) or Ca^{2+} mobilization (Chen et al., 1997; Yu et al., 1998; Wong et

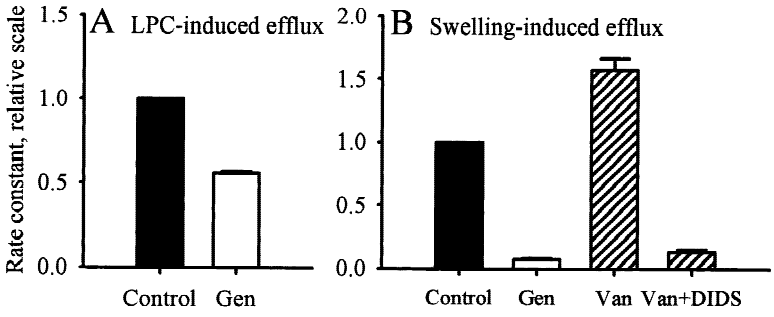

Fig. 6. Effect of tyrosine phosphorylation on the LPC-induced and the swelling-induced taurine efflux. HeLa cells, grown to 80% confluence, were serum starved and preloaded with [^{14}C]-taurine for 2 hr in NaCl medium containing 5 mM glucose. The efflux experiments were performed in either isotonic KCl medium with addition of 10 μM LPC, with palmitic acid in *sn*-1, or isotonic KCl with a shift to hypotonic KCl medium. Genistein (Gen, 100 μM) was added 30 min before and present during the efflux experiment. Vanadate (Van, 200 μM) and DIDS (100 μM) were present in the efflux media throughout the efflux experiment. The maximal rate constant following addition of LPC (*A*) or hypotonic exposure (*B*) was estimated in the absence (control) and presence of inhibitors. Values are given relative to control ± SEM and represent 3 (LPC, genistein), 4 (hypotonic, genistein), 10 (hypotonic, vanadate) and 3 (hypotonic, vanadate plus DIDS) sets of paired experiments. The effect of genistein on the LPC-induced and swelling-induced taurine efflux was significant at a 0.02 and 0.005 level, respectively. The effect of vanadate was significant at a 0.0001 level.

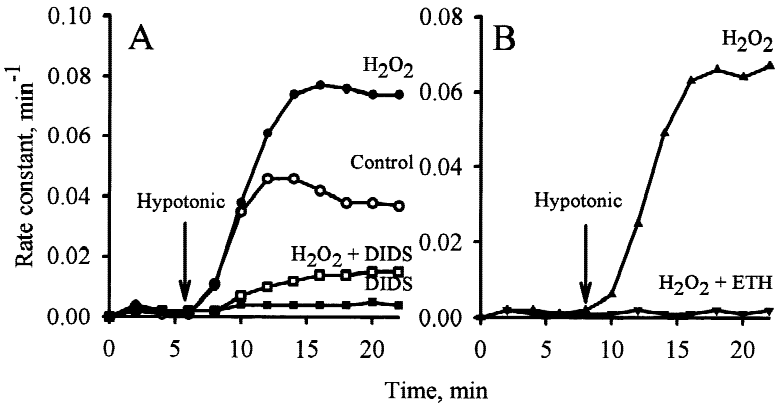

Fig. 7. Effect of H_2O_2 on the swelling-induced taurine efflux. HeLa cells were grown to 80% confluence, serum starved and preloaded with [^{14}C]-taurine for 2 hr in NaCl medium containing 5 mM glucose. The efflux experiments were performed in isotonic KCl medium with a shift to hypotonic KCl medium as indicated by the arrow. H_2O_2 (2 mM), DIDS (100 μM, *A*) and ETH 615-139 (10 μM, *B*) were present in the efflux media throughout the efflux experiment. The curves represent 8 (H_2O_2), 6 (DIDS), 3 (H_2O_2 plus DIDS), and 3 (ETH 615-139 plus DIDS).

al., 1998; Golfman et al., 1999). The data presented in Fig. 2 and Table 2 demonstrate that addition of LPC, containing the saturated palmitic acid, to HeLa cells suspended in isotonic solution results in a transient, concentration-dependent increase in the taurine efflux. LPC containing a longer saturated fatty acid (stearic acid) or a mono-unsaturated fatty acid (oleic acid) also induce a transient but smaller increase in the taurine release. LPI, LPS, which have negatively charged head groups, LPE, which has a smaller head group than LPC, and LPA are all relatively ineffective as taurine releasing agents during isotonic conditions. Similarly, it was demonstrated that LPC species with palmitic or stearic acid enhanced PLA_2-mediated release of arachidonic acid in human endothelial cells (Wong et al., 1998) and cardiac myoblastic H9c2 cells (Golfman et al., 1999), while LPE, LPS, LPI and LPA had almost no effect. The haemolytic potency of lysophospholipids is also determined by the saturation and size/length of the aliphatic chain, i.e., saturated chains with 16–18 carbon atoms give optimal lytic activity (*see* Weltzien, 1979).

The observations that LPC at high concentrations (25 μM) increases the release of creatine kinase from cardiomyocytes (Chen et al., 1997) as well as nucleotides from HeLa cells (Fig. 3) and cardiomyocytes (Takahashi et al., 2000), and that the effect of LPC on taurine and nucleotide release in HeLa cells is mimicked by the permeabilizing agent digitonin (*see* Fig. 3 and Table 2) support the notion that addition of LPC at these concentrations leads to a general breakdown of the membrane permeability barrier. Lysophospholipids are cone shaped, i.e., the cross-sectional area of the acyl chain is smaller than that of the polar head group, and accumulation of lysophospholipids in a membrane stabilizes convex surfaces and affects the energetic costs of a membrane deformation and the conformation state of membrane proteins (*see* Lundbæk & Andersen, 1994). However, the hypothesis that the effect of lysophospholipids is an uncontrolled effect of a membrane permeabilizing agent is opposed by the specific effects of LPC on signal transduction (*see above*) and by the observation, as already intimated by Wong and coworkers (1998), that only LPC species release arachidonic acid (Wong et al., 1998; Golfman et al., 1999) and taurine (Fig. 2 and Table 2), whereas LPS, LPI, LPA and LPE, despite their detergent properties, are more or less ineffective.

It has been demonstrated that LPC increases $[Ca^{2+}]_i$ in a concentration-dependent manner (2.5 to 10 μM range) in rat cardiomyocytes and that the increase reflects Ca^{2+} influx as well as Ca^{2+} release from the sarcoplasmatic reticulum (Yu et al., 1998). An increase in $[Ca^{2+}]_i$ will in itself affect Ca^{2+}-dependent enzymes ($cPLA_2$, 5-LO, calmodulin regulated systems etc.) as well as Ca^{2+}-sensitive ion transporting systems (K^+ and Cl^- channels) (*see* Hoffmann & Mills, 1999). Increasing $[Ca^{2+}]_i$ does not elicit taurine efflux from HeLa cells suspended in isotonic medium (Table 2), whereas LPC, when added at low concentrations (5–10 μM), induces taurine loss without any consistent increase in $[Ca^{2+}]_i$ (*see* Results). It is, therefore, unlikely that the LPC-induced taurine release in HeLa cells should be Ca^{2+} mediated. It is noted that even though the volume regulatory response in e.g., Ehrlich cells takes place without any detectable increase in $[Ca^{2+}]_i$, an increase in $[Ca^{2+}]_i$ will increase the rate of the volume regulatory response (Jørgensen et al., 1997). Similarly, the swelling-induced taurine release in HeLa cells is potentiated following an increase in $[Ca^{2+}]_i$ (Fig. 1*B*, Table 1; Lambert & Sepúlveda, 2000). LPC has been shown to release ATP in rat cardiomyocytes (Takahashi et al., 2000) and in HeLa cells (Fig. 3), and ATP is known to bind to nucleotide receptors and mobilize Ca^{2+} in various cell types. However, ATP has no significant effect on taurine release in HeLa cells suspended in isotonic medium (Lambert & Sepúlveda, 2000), excluding that the effect of LPC seen during isotonic conditions is secondary to an increased release of ATP.

The opening question was whether lysophospholipids could play a role in the swelling-induced activation of the taurine efflux pathway. In Ehrlich cells it has been estimated that the production of LPC from PC with stearic acid in the *sn*-1 position increases by 0.04%-point within the first 145 seconds following exposure to a hypotonic solution with half original osmolarity (Thoroed et al., 1997). In a rough estimate a similar shift in the LPC production in HeLa cells would result in a cytosolic LPC concentrations at about 6 μM [HeLa cells contain 6 μl cell water per mg protein (Ikehara et al., 1992); 0.67 mg phospholipids per mg protein (Gennis, 1989); PC constitutes 70% of the phospholipids (Baburina & Jackowski, 1999) among which 10% contain arachidonic acid in the *sn*-2 position (Sagar & Das, 1995), i.e., 7% of the phospholipids is substrate for $cPLA_2$; FW for LPC is 495.6]. As LPC has a high affinity for hydrophobic proteins and mammalian cells have a large capacity for reacylation of lysophospholipids it seems reasonable to assume that the cytosolic LPC concentration following hypotonic exposure does not reach the critical concentration that elicits the effects reported in the paper. Thus, LPC is not regarded as a lipid second messenger in the activation of the volume sensitive taurine efflux pathway in HeLa cells. This notion is supported by the observation that the LPC-induced taurine efflux pathway differs from the swelling-induced taurine efflux pathway with respect to sensitivity towards serum starvation (Lambert & Sepúlveda, 2000) and channel blockers (Fig. 4). Furthermore, activation of taurine efflux by cell swelling involves 5-LO activity and is modulated by protein tyrosine kinase/phosphatase activity, whereas activation of taurine efflux by LPC involves generation of superoxides, phosphorylation of tyrosine residues but apparently not production of 5-LO products (Figs. 4–7). In this context it should be noted that lysophospholipids, e.g., LPA not only acts as a Ca^{2+} mobilizing agent in many cell types (*see* Moolenaar et al., 1997; Goetzl & An, 1998) but in the case of HeLa cells raises the intracellular concentration of reactive oxygen species (ROS) and that these ROS mediate tyrosine phosphorylation of the epidermal growth factor (EGF) receptor as well as activation of mitogen-activated protein kinase (Cunnick et al., 1998). On the other hand, Mongin and coworkers (1999) have recently demonstrated that osmotic swelling leads to activation of at least two taurine efflux pathways in primary astrocyte cultures and that only one of these pathways is regulated by tyrosine phosphorylation, i.e., by a tyrphostin-sensitive protein tyrosine kinase different from the EGF receptor kinase. Protein tyrosine phosphorylation is also involved in the activation of volume sensitive ionic conductances in a process that involves reorganization of the F-actin cytoskeleton and the p125 focal adhesion kinase ($p125^{FAK}$; Tilly et al., 1996). As growth factors and hormones stimulate tyrosine phosphorylation of $p125^{FAK}$ the observation that serum starvation leads to reduction in the swelling-induced taurine efflux (Lambert & Sepúlveda, 2000) could point to a role of $p125^{FAK}$ in the regulation of the volume sensitive taurine efflux pathway. Investigations to elucidate the nature of the protein tyrosine kinases involved in swelling-induced and LPC-induced taurine efflux are in progress.

LPC is, as indicated in the Introduction, released during ischemia and exogenous LPC inflicts injury on heart cells similar to that induced by ischemia and reperfusion (Hoque, Haist & Karmazyn, 1997). Ischemic rat cardiomyocytes release taurine, ATP as well as creatine phosphokinase, and they exhibit morphological degeneration and beating cessation (Takahashi et al., 2000). However, the effects in the ischemic rat cardiomyocytes are reduced in the presence of 20 mM taurine (Takahashi et al., 2000). Taurine is known to bind to phospholipids (*see* Huxtable, 1992) and it is therefore possible that taurine released by LPC buffers exogenous LPC and thereby reduces the LPC availability. Whether taurine released from ischemic cells (Takahashi et al., 2000) actually protects the cell from the concomitant LPC-induced breakdown of the membrane permeability barrier is an interesting question for future investigations.

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