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Reactive Oxygen Species Regulate Swelling-induced Taurine Efflux in NIH3T3 Mouse Fibroblasts

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Abstract. NIH3T3 mouse fibroblasts generate reactive oxygen species (ROS) and release taurine following exposure to hypotonic medium and to isotonic medium containing the lipase activator melittin. The swelling-induced taurine release is potentiated by H_2O_2, the calmodulin antagonist W7, and ATP, but inhibited by the antioxidant butulated hydroxytoluene (BHT), the NAD(P)H oxidase inhibitor diphenylene iodonium (DI), and the iPLA$_2$ inhibitor bromoenol lactone (BEL). The swelling-induced ROS production is also inhibited by BHT and BEL. H_2O_2 does not affect the volume set point for activation of the volume-sensitive taurine efflux. The 5-lipoxygenase (5-LO) inhibitor ETH 615-139 impairs the swelling-induced taurine efflux in the absence as well as in the presence of H_2O_2. The melittin-induced taurine release is, in analogy with the swelling-induced taurine release, potentiated by H_2O_2 and inhibited by BHT, DI, BEL, ETH 615-139 and anion channel blockers. Thus, swelling- and melittin-induced cell signalling and taurine release involve joint elements. The swelling-induced taurine efflux is potentiated by the protein tyrosine phosphatase inhibitor vanadate, and the potentiating effect of H_2O_2 and vanadate is impaired in the presence of protein tyrosine kinase inhibitor genistein. It is suggested that (i) iPLA$_2$ and 5-LO activity is required for the swelling-induced activation of taurine efflux from NIH3T3 cells, (ii) ROS are produced subsequent to the PLA$_2$ activation by the NAD(P)H oxidase complex, and (iii) ROS inhibit a protein tyrosine phosphatase (PTP1B) causing a potentiation of the swelling-induced taurine release.

Key words: Volume regulation — Focal adhesion kinase — PP2

Introduction

The ability to control the cell volume is essential for the control of cellular function and processes (Lang et al., 1998). NIH3T3 fibroblasts exposed to hypotonic solutions swell due to osmotic water uptake; they reach a maximal degree of cell swelling within 1 min, whereupon they regulate their volume towards their initial value (Pasantes-Morales et al., 1997). The back regulation is due to net loss of KCl and organic osmolytes (Moran et al., 1997; Pasantes-Morales et al., 1997). The K^+ efflux induced by the cell swelling is mediated in part by a K^+-selective pathway and in part by K^+, Cl^- cotransport (Pasantes-Morales et al., 1997; Pedersen et al., 2002). The Cl^- efflux is via a nonselective anion pathway (Pasantes-Morales et al., 1997), and patch-clamp studies have revealed that the Cl^- current evoked by cell swelling in NIH3T3 fibroblasts is Ca^{2+}-independent and exhibits moderate outward rectification as well as time-dependent inactivation (Pedersen et al., 2002). The swelling-induced organic osmolyte efflux pathway accepts neutral amino acids including taurine, but apparently not basic amino acids (Pasantes-Morales et al., 1997). The cellular concentration of free amino acids in NIH3T3 cells is close to 45 mM and it has been estimated that the free amino acids constitute 20% of the total osmolyte loss during the RVD response following exposure to a 50% hypoosmotic solution (Moran et al., 1997). The cellular taurine concentration in NIH3T3 cells is estimated at 10 mM and about 90% of the taurine diffuses out of the NIH3T3 cell within 15 minutes following exposure to the 50% hypoosmotic solution (Moran et al., 1997). The swelling-induced taurine release from NIH3T3 cells is Na^+-independent and mediated via a leak pathway that is blocked by 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB), 1,9-dideoxyforskolin (DDF), 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS), and

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by polyunsaturated fatty acids (arachidonic acid, linoleic acid; Moran et al., 1997). The time course for the swelling-induced taurine efflux differs from the time course for the swelling-induced Cl^- efflux, i.e., the taurine efflux peaks within 3 to 4 min following hypotonic exposure, whereas the Cl^- release peaks within 40 seconds and ends within 2 min (Moran et al., 1997). Furthermore, the swelling-induced Cl^- loss and the swelling-induced taurine loss diverge with respect to their sensitivity to DIDS (Moran et al., 1997), Rho kinase inhibitors and expression of constitutively active Rho (Pedersen et al., 2002). Loss of taurine and Cl^- via separate pathways has previously been demonstrated for Ehrlich ascites tumour cells (Lambert & Hoffmann, 1993) and for HeLa cells (Stutzin et al., 1999).

The signal cascade, which is activated by osmotic cell swelling and which leads to activation of osmolyte efflux from mammalian cells, has been reported to involve various elements, including the cytoskeleton, phospholipases, lipoxygenases, protein tyrosine kinases/phosphatases, GTP-binding proteins, Ca^{2+} calmodulin, as well as second messengers such as nucleotides and eicosanoids. Although the cellular F-actin content is generally decreased following cell swelling and interference with cytoskeletal components affects the volume regulatory response, it has not yet been unequivocally demonstrated whether or where the F-actin interferes with the cell volume recovering processes (Pedersen, Hoffmann & Mills, 2001). Phospholipase A_2 (PLA_2), which catalyzes the formation of arachidonic acid and lysophospholipids, as well as lipoxygenases, which oxidize arachidonic acid, are essential/permissive elements of the swelling-induced activation of taurine efflux in various cells (Lambert & Hoffmann, 1993; Margalit et al., 1993a; Light et al., 1997; Basavappa et al., 1998; Lambert & Sepulveda, 2000). Hypotonic exposure triggers a rapid and transient increase in the tyrosine phosphorylation of several proteins in human intestine 407 cells (Tilly et al., 1993) and an enhanced tyrosine phosphorylation shifts the osmosensitivity of the volume-sensitive taurine efflux system in supraoptic astrocytes (Deleuze et al., 2000) and interferes with the closing of the volume-sensitive taurine efflux pathways in NIH3T3 cells (Pedersen et al., 2002). Inhibition of GTP-binding proteins reduces the swelling-induced volume regulatory process in Ehrlich ascites tumor cells (Thoroed et al., 1997), whereas expression of constitutively active RhoA (RhoAV14) accelerates the rate of the volume regulatory process in NIH3T3 cells by increasing the activity of the volume-sensitive K^+, Cl^-, and taurine efflux pathways (Pedersen et al., 2002). Inhibition of the Ca^{2+}/calmodulin complex impairs swelling-induced taurine efflux in Ehrlich cells (Lambert & Hoffmann, 1993) and HeLa cells (Kirk & Kirk, 1994; Lambert & Sepulveda, 2000) and there seems to be a close relationship between the ability of a drug as a Ca^{2+}/calmodulin antagonist and its ability to block the swelling-induced taurine efflux from HeLa cells (Kirk & Kirk, 1994). Few second messengers have been assigned a role in the swelling-induced release of osmolytes. ATP is released from Ehrlich ascites tumor cells by mechanical stress (Pedersen et al., 1999) and ATP, released in response to hypotonic exposure, is reported to act as an autocrine activator of the swelling-induced Cl^- channels in rat hepatoma cells (Wang et al., 1996). Arachidonic acid, released by PLA_2, is recognized as an intracellular messenger per se as well as a substrate for the eicosanoid-generating enzymes, i.e., the 5-lipoxygenase (5-LO) and the constitutive active and the inducible cyclooxygenases (COX-1, COX-2). Oxidation of arachidonic acid via the 5-LO to leukotriene D_4 (LTD_4) is required for activation of the volume-sensitive taurine efflux pathway (Lambert & Hoffmann, 1993; Lambert, 1998) and the volume-sensitive K^+ efflux pathway (Hoffmann, 1999) in Ehrlich ascites tumor cells, whereas oxidation of the fatty acid via the 12-lipoxygenase to hepoxilin A_3 is required for activation of the volume regulatory response in swollen human blood platelets (Margalit et al., 1993b). The eicosanoid 5-HETE has been considered as a second messenger in the swelling-induced activation of the taurine efflux in HeLa cells (Lambert & Sepulveda, 2000).

The initial signal that triggers the volume regulatory signalling cascade in mammalian cells by osmotic cell swelling is not known. PLA_2 appears as an upstream, initial element of the swelling-induced signalling cascade, and distortion of the lipid bilayer (Lehtonen & Kinnunen, 1995) and an increase in reactive oxygen species (ROS) are known to affect PLA_2 activity (Farber & Young, 1981; Lipton, 1999; Martinez & Moreno, 2001; Balboa & Balsinde, 2002). The present work was, therefore, initiated to investigate (i) whether PLA_2 activity is required for the swelling-induced activation of taurine efflux in NIH3T3 cells and (ii) whether ROS represent an important element in the initiation or regulation of the swelling-induced cell signalling that leads to taurine release.

Materials and Methods

CHEMICALS

Growth media, antibiotics, sera and trypsin were from Life Technologies (Denmark). All compounds were, if not otherwise stated, from Sigma (St. Louis, MO). The concentration of the stock solutions is given in parentheses. [^{14}C]-taurine was from NEN Life Science Products, Inc. (Boston, MA). 5-(and-6)-carboxy-2′, 7′-dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA, 50 mM, Molecular Probes, Leiden, The Netherlands), bromoenol lactone (BEL, 10 mM), 4-[(Bromophenyl)amino]-6,7-dimethoxy quinazoline (PD153035, 50 μM, Calbiochem, San Diego, CA), 4-amino-5-(4-chlorophenyl)-7-(t-butyl) pyrazolo (3,4-d)pyridine (PP2, 5 μM, Calbiochem), and 4-amino-7-phenylpyrazol(3,4) pyrimidine (PP3, 5 μM, Calbiochem) were dissolved in dimethylsulfoxide. ATP was prepared as 10.7 mM stock solution in NaCl medium containing 0.1 mM EGTA. Arachidonic acid (50 mM), ETH 615-139 (4 mM, donated by Dr. I. Ahnfelt-Rønne, Løvens Kemiske Fabrik, Denmark), butulated hydroxytoluene (BHT, 400 mM), genistein (10 mM), 4,4′-diisothiocyano-2, 2′-stilbene acid (DIDS, 20 mM), and diphenylene iodonium (DI, 10 mM), were dissolved in ethanol. N-(6-aminohexyl)-5-chloro-1-naphathalene sulphonamide (W7, 5 mM), melittin (1 mg/ml), and NaVanadate (Na_3VO_4, 20 mM) were dissolved in H_2O. N-acetyl-L-cystein was dissolved in growth medium. Epidermal growth factor (EGF, 10 μg/ml) was prepared in NaCl medium containing 0.2% bovine serum albumin.

INORGANIC MEDIA

The phosphate buffered saline (PBS) contained (in mM) 137 NaCl, 2.6 KCl, 6.5 Na_2HPO_4, and 1.5 KH_2PO_4. Isosmotic NaCl medium contained 143 NaCl, 5 KCl, 1 Na_2HPO_4, 1 $CaCl_2$, 0.1 $MgSO_4$, 5 glucose, and 10 HEPES. Isoosmotic KCl medium contained 150 KCl, 1.3 $CaCl_2$, 0.5 $MgCl_2$, and 10 HEPES. Hypoosmotic NaCl or KCl solutions were obtained by reduction of the NaCl or KCl in the isoosmotic solutions to 95 mM, with the other components remaining unchanged. pH was in all solutions adjusted at 7.40.

CELL CULTURES

The mouse fibroblast cell line NIH3T3 (clone 7) was maintained as a monolayer culture in Dulbecco's Modified Eagle Medium (high glucose) (DMEM) containing heat-inactivated fetal bovine serum (10%) and penicillin (100 units/ml). Incubation temperature was 37°C and CO_2 was 5%. The cell cultures were split every 3–4 days using 0.25% trypsin in PBS to detach the cells.

ESTIMATION OF ROS PRODUCTION

Cells were cultured for 24 hr on HCl and ethanol-washed glass coverslips (10–50 mM). Confluence at the time of experiment was 80%. For ROS measurements, the cells were washed 2 times with PBS and subsequently incubated for 2 hr with serum-free DMEM containing the fluorescent probe carboxy-H_2DCFDA (20 μM). Carboxy-H_2DCFDA is taken up by the cells, deacetylated by intracellular esterases and then rapidly oxidized by various reactive oxygen species to a fluorescent, detectable compound. An increase in the fluorescence is accordingly taken as an indication of formation of the entire group of ROS. The cells were washed with isotonic solution and the coverslips subsequently placed vertically in a polystyrene cuvette (10 mM path length, 50 degree angle relative to the excitation light) containing experimental solution, and analysis was performed on a PTI Ratio Master spectrophotometer. The experimental solution in the cuvette was continuously stirred by use of a teflon-coated magnet driven by a motor attached to the cuvette house. The excitation and emission wavelengths were 490 and 515 nm, respectively, and data were collected every 2 sec. It is noted that the ROS-dependent fluorescence depends on cell density, dye concentration and incubation time. Consequently, the effect of reduction in osmolarity and addition of diverse agents are presented as and evaluated from time traces obtained from the same cell preparation (*see* e.g., Figs. 2 and 4).

EFFLUX MEASUREMENTS AND ESTIMATION OF RATE CONSTANTS

Taurine efflux measurements were performed as described previously (Hall et al., 1996). Briefly, cells grown to 80% confluence in 6-well polyethylene dishes were loaded for 2 hr in DMEM containing [^{14}C]-taurine (80 nCi/ml). Prior to the efflux experiment, the preincubation solution was aspirated and the cells were washed 5 times with 1 ml isosmotic solution to remove excess extracellular [^{14}C]-taurine. After the final wash, 1 ml of experimental solution was added to the dish, left for 2 min, and transferred to a scintillation vial for estimation of ^{14}C activity (β-scintillation counting, Ultima Gold^{TM}). This procedure was repeated for 20 to 30 min. At the end of the experiment, the [^{14}C]-taurine activity remaining inside the cells was estimated by lysing the cells with 1 ml NaOH (0.5 M, 1 hr), washing the dishes 2 times with distilled water and estimating the ^{14}C activity in the NaOH lysate as well as in both water washouts. The total ^{14}C activity in the cell system was estimated as the sum of activity in all the efflux samples and the intracellular activity. The natural logarithm to the fraction of ^{14}C activity remaining in the cells at a given time was plotted versus time, and the rate constant for the taurine efflux at each time point was estimated as the negative slope of the graph between the time point and the proceeding time point.

STATISTICAL ANALYSIS

Data are presented either as individual experiments, representative of at least three independent sets of experiments or as mean values ± standard error of the mean (SEM). Statistical significance was estimated by Student's *t*-test. For all statistical evaluations, P values <0.05 were taken to indicate a significant difference, n is the number of experiments.

Results

iPLA$_2$ AND 5-LIPOXYGENASE ARE INVOLVED IN THE SWELLING-INDUCED ACTIVATION OF TAURINE EFFLUX IN NIH3T3 FIBROBLASTS

Exposure to hypotonic solution has previously been demonstrated to induce taurine release from NIH3T3 cells (Pasantes-Morales et al., 1997; Pedersen et al., 2002). This is confirmed in Fig. 1, where it is seen that exposure to hypotonic NaCl medium (200 mOsm) results in a transient increase in the rate constant for taurine efflux. The maximal rate constant for the swelling-induced taurine efflux upon hypotonic (200 mOsm) exposure was estimated at 0.029 ± 0.001 min^{-1} (n = 127). From Fig. 1*A* it is seen that the swelling-induced taurine efflux is reduced in the presence of bromoenol lactone (BEL), a relative specific blocker of the Ca^{2+}-independent PLA$_2$ (iPLA$_2$, Balsinde et al., 1999) and potentiated in the presence of the calmodulin antagonist W7. It is estimated that the maximal rate constant for the swelling-induced taurine efflux is significantly reduced by about 40% and 70% in the presence of 10 μM and 30 μM BEL, respectively (Table 1) and significantly increased 2.04 ± 0.09 fold (n = 3 paired set of experiments) in the presence of 50 μM W7. As binding of calmodulin to iPLA$_2$ results in loss of enzyme activity (Jenkins et al., 2001) it seems reasonable to suggest that iPLA$_2$ is involved in the swelling-induced activation of taurine release from NIH3T3 cells.

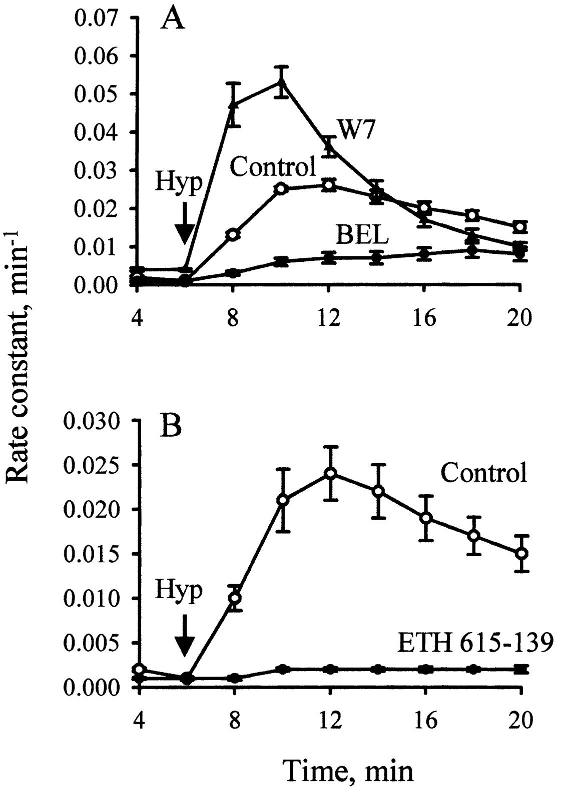

Fig. 1. Effect of the iPLA$_2$ inhibitor bromoenol lactone, the calmodulin antagonist W7, and the 5-lipoxygenase inhibitor ETH 615-139 on the swelling-induced taurine efflux from NIH3T3 mouse fibroblasts. Cells, grown to 80% confluence, were loaded with [^{14}C]-taurine for 2 hr in DMEM. The cells were washed and the efflux experiments subsequently performed in NaCl medium with a shift in osmolarity from 300 mOsm to 200 mOsm 6 min after initiation of the efflux experiment. The rate constant for the taurine efflux was calculated and plotted versus time. The arrow indicates the shift in osmolarity. (*A*) BEL (30 μM, *filled circles*) was added to the loading medium 30 min before initiation of the efflux experiments and present throughout the whole experimental period. W7 (50 μM, *triangles*) was present throughout the whole efflux experiment. (*B*) ETH 615-139 (10 μM, *filled circles*) was included in the efflux medium throughout the whole release experiment. Control cells (*empty circles*) were not exposed to BEL, W7 or ETH 615-139. Rate constants are given as mean values ± SEM of 10 (control), 10 (BEL) and 5 (W7) sets of experiments in *A*, and of 9 (control) and 9 (ETH 615-139) sets of experiments in *B*.

Addition of AACOCF$_3$, a reversible and slow-binding inhibitor of the cytosolic, Ca^{2+}-dependent PLA$_2$ (cPLA$_2$, Balsinde et al., 1999), has no significant effect on the swelling-induced taurine efflux from NIH3T3 cells (Table 1). From Fig. 1*B* it is seen that addition of ETH 615-139, which blocks the 5-LO directly (Kirstein Thomsen & Ahnfelt-Ronne, 1991), essentially abolishes the swelling-induced taurine efflux from NIH3T3 cells, i.e., the maximal rate constant for the swelling-induced taurine efflux is reduced by 90% in the presence of 10 μM ETH 615-139 (Table 1). The anion channel blockers DIDS and arachidonic acid reduce the rate constant of the swelling-induced taurine efflux by 80% (Table 1), which is in accordance with previously published data (Moran et al., 1997). It is noted that arachidonic acid only inhibits the taurine efflux at a concentration above 5 μM, whereas at 1 μM it has no significant effect on the swelling-induced efflux (Table 1). Based on these findings it is suggested that sequential activation of iPLA$_2$ and 5-LO is required for activation of the volume-sensitive taurine efflux in NIH3T3 cells.

ROS Production and Taurine Release upon Cell Swelling Occur downstream to PLA$_2$ Activation

ROS are reported to enhance the activity of iPLA$_2$ in macrophages (Martinez & Moreno, 2001) and the experiments presented in Figs. 2 and 3 were performed to see whether ROS are produced in NIH3T3 cells upon hypotonic exposure and whether ROS regulate the volume-sensitive taurine efflux. From Figs. 2*A* and *B* it is seen that there is a minor production of ROS in NIH3T3 cells under isotonic conditions and that exposure to H_2O_2 (2 mM) or hypotonic NaCl medium (200 mOsm) elicits an increase in the ROS production. The swellings-induced ROS production is reduced in the presence of the antioxidant BHT (0.5 mM, Fig. 2*C*) and the iPLA$_2$ inhibitor BEL (30 μM, Fig. 2*D*). Thus, ROS are generated following osmotic cell swelling and most probably at a step downstream to iPLA$_2$ activation. From Figs. 3*A* and *B* it is seen that the swelling-induced taurine efflux is potentiated in the presence of H_2O_2 and reduced in the presence of BHT. It is estimated that the maximal rate constant for taurine efflux obtained by osmotic cell swelling in hypotonic NaCl medium (200 mOsm) is increased about 4- to 5-fold in the presence of 2 mM H_2O_2 and reduced by 75% in the presence of 0.5 mM BHT (Table 1). Addition of H_2O_2 to NIH3T3 cells under isotonic conditions does not affect the rate constant for taurine efflux, i.e., the rate constant for the taurine efflux following 20 min exposure to 2 mM H_2O_2 is estimated at 0.0015 ± 0.0002 min^{-1} (n = 3), which is to be compared with 0.0016 ± 0.0003 min^{-1} (n = 11) for control cells. Exposing the NIH3T3 cells to *N*-acetylcystein (NAG, 20 mM for 24 hr), which increases the intracellular level of reduced gluthation and thereby elimination of ROS via the gluthation peroxidase system, reduces the maximal rate constant for the swelling-induced taurine efflux marginally but significantly by 10% (Table 1). From Fig. 3*C* it is seen that exposure of NIH3T3 cells to 25 μM NAD(P)H oxidase inhibitor diphenylene iodonium (DI), which would be expected to reduce ROS production, does not affect the initiation of the swelling-induced efflux pathway but accelerates dramatically its inactivation. However, H_2O_2 is still able to potentiate taurine ef-

Table 1. Pharmacological profile of the swelling- and the melittin-induced taurine efflux from NIH3T3 mouse fibroblasts

Effector	Concentration	Taurine efflux maximal rate constant[1]			
		Hypotonic	*n*	Isotonic + Melittin	*n*
Control		1		1	
BEL	10 μM	0.62 ± 0.05*	5	—	
	30 μM	0.29 ± 0.05*	11	0.10 ± 0.02*	5
AACOCF3	40 μM	1.13 ± 0.33*	3	0.43 ± 0.08*	3
ETH 615-139	10 μM	0.10 ± 0.02*	9	0.07 ± 0.01*	3
BHT	0.5 mM	0.24 ± 0.03*	6	0.70 ± 0.13*	6
H_2O_2	2 mM	4.62 ± 0.21*	23	1.80 ± 0.12*	6
NAC	20 mM	0.90 ± 0.01	3	0.31 ± 0.10*	3
Arachidonic acid	1 μM	1.31 ± 0.20	5	—	
	10 μM	0.20 ± 0.03*	3	—	
	50 μM	0.23 ± 0.05*	3	0.14 ± 0.06*	3
DIDS	100 μM	0.19 ± 0.01*	4	0.02 ± 0.01*	3
ATP		2.56 ± 0.14*	5	1.55 ± 0.10*	3

[1] Rate constants measured in the presence of effectors are given relative to the value found in the respective control cells.

The maximal rate constant for taurine efflux obtained by either hypotonic exposure (200 mOsm) or stimulation under isotonic conditions with melittin (0.5 μg/ml) was estimated in NaCl media as outlined in Figs. 1 and 4*B*. NAC and BEL were added to the cells 24 hr and 30 min, respectively, before initiation of the efflux experiments and present throughout the whole experimental period. AACOCF3, ETH 615-139, BHT, H_2O_2, and the anion channel blocker arachidonic acid and DIDS were included in the efflux media during the whole experimental period. ATP was present from the time of hypotonic exposure or addition of melittin. Melittin was increased from 0.5 μg/ml to 1 μg/ml in the DIDS and arachidonic acid experiments, *n* is the number of paired experiments. Asterix indicates that the rate constant is significantly different from the control value.

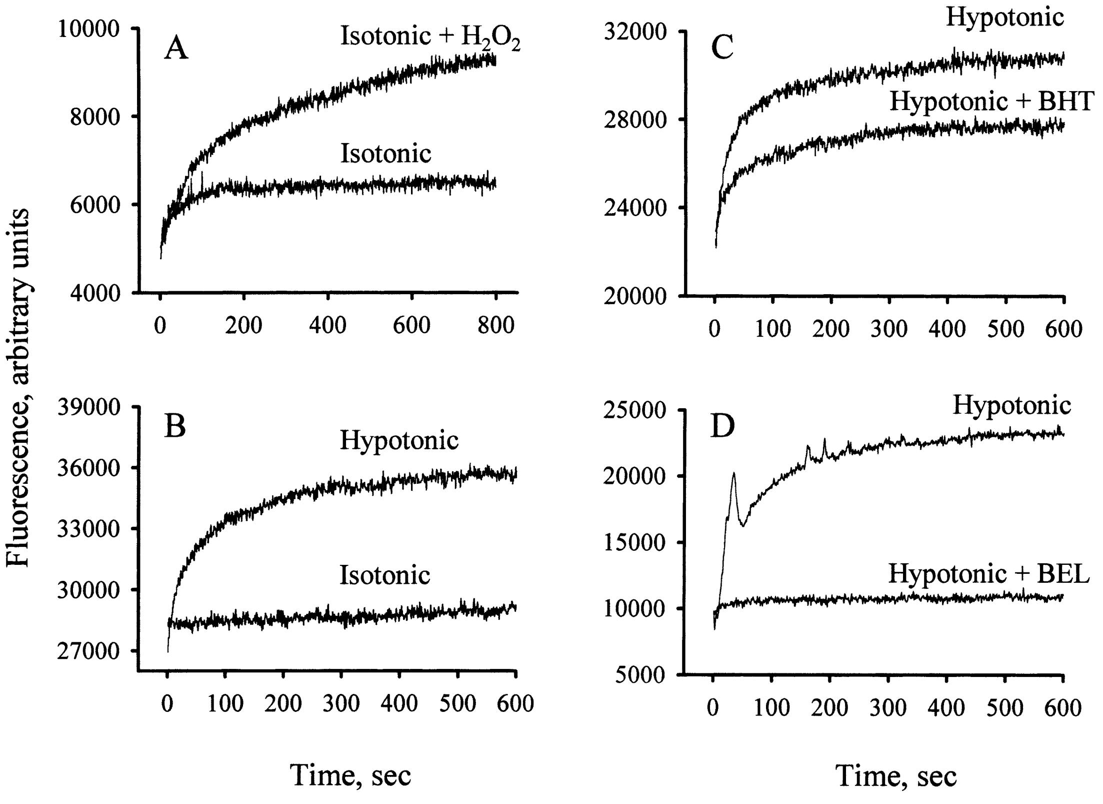

Fig. 2. ROS production in NIH3T3 mouse fibroblasts following addition of H_2O_2 or hypotonic exposure. Cells, grown on coverslips (80% confluence), were loaded with the fluorescent, ROS-sensitive probe carboxy-H_2DCFDA. The cells were washed and at time zero exposed to isotonic (300 mOsm) or hypotonic NaCl (200 mOsm) media, and ROS production was followed with time as outlined in Material and Methods. (*A*) Cells transferred to isotonic NaCl medium with/without H_2O_2 (2 mM). (*B*) Cells transferred to isotonic or hypotonic medium. (*C*) Cells transferred to hypotonic medium with or without BHT (0.5 mM). (*D*) Cells transferred to hypotonic medium with or without BEL (30 μM). All traces are representative of at least 3 sets of experiments.

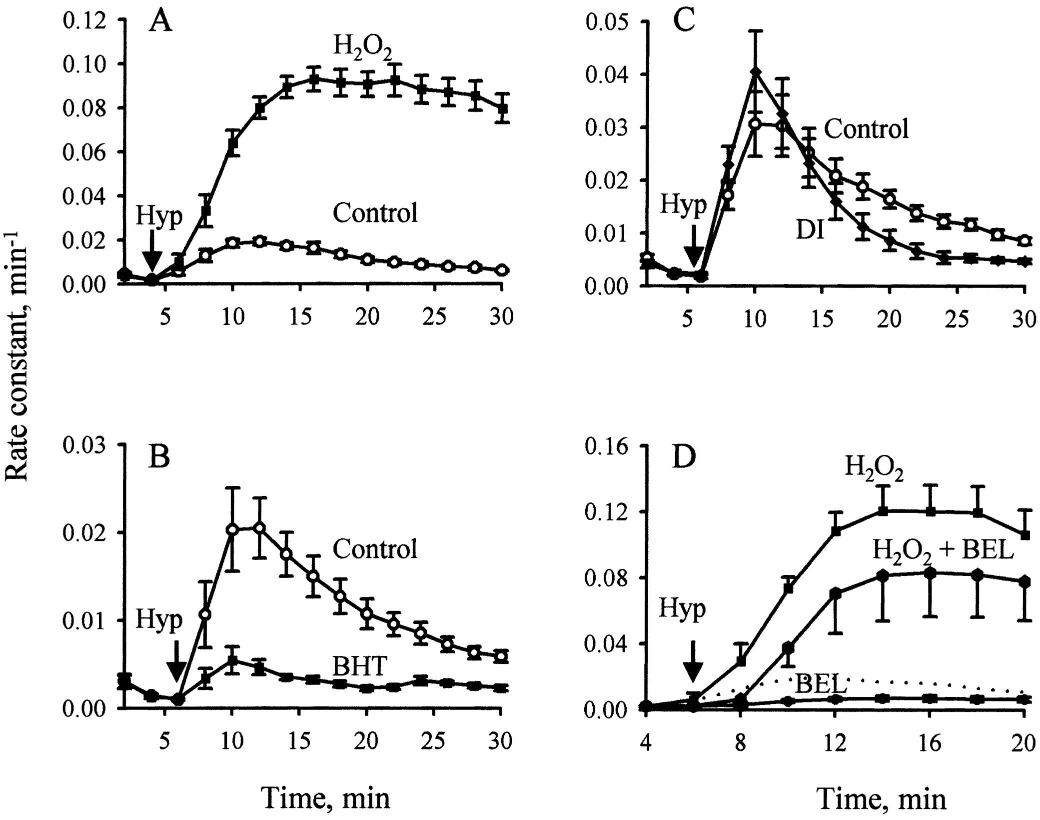

Fig. 3. Effect of H_2O_2 and the antioxidant BHT on the swelling-induced taurine efflux from NIH3T3 mouse fibroblasts. Cells, grown to 80% confluence, were loaded with [^{14}C]-taurine for 2 hr in DMEM. The cells were washed and the efflux experiments subsequently performed in isotonic NaCl medium with a shift in osmolarity from 300 to 200 mOsm. The rate constant for the taurine efflux was calculated and plotted versus time. The arrow indicates the time of shift in osmolarity. (*A*) Control cells (*empty circles*) and cells exposed to H_2O_2 (2 mM, *squares*) throughout the whole efflux experiment. (*B*) Control cells (*empty circles*) and cells exposed to BHT (0.5 mM, *squares*) throughout the whole efflux experiment. (*C*) Control cells (*empty circles*) and cells exposed to DI (25 μM, *diamonds*). DI was added to the loading medium 30 min before initiation of the efflux experiments and present throughout the whole experimental period. (*D*) Cells exposed to H_2O_2 (2 mM, *squares*), to BEL (30 μM, *filled circles*) and to H_2O_2 plus BEL (*hexagons*). Dotted line represents the control cells from (*A*). Values in *A* to *D* represent 9, 6, 5 and 6 sets of paired experiments, respectively.

flux even in the presence of the iPLA$_2$ inhibitor BEL (Fig. 3*D*). Thus, ROS production in NIH3T3 cells upon hypotonic exposure seems to involve NADPH-dependent reduction of oxygen via the NAD(P)H oxidase and ROS modulate the concomitant taurine efflux.

MELITTIN INDUCES TAURINE EFFLUX UNDER ISOTONIC CONDITIONS

Addition of 1 μg per ml of the lipase activator melittin (≈ 0.35 μM; MW = 2846.5) to NIH3T3 cells under isotonic conditions leads to a significant ROS production (Fig. 4*A*) as well as a transient increase in taurine release from NIH3T3 cells with a similar time course as the one seen after hypotonic exposure (Fig. 4*B*). The maximal rate constant for taurine efflux following exposure to 0.5 and 1.0 μg melittin per ml is estimated at 0.043 ± 0.003 min^{-1} ($n = 48$) and 0.165 ± 0.009 min^{-1} ($n = 14$), respectively. Increasing the melittin concentration to 5 or 10 μg per ml depleted the cells for ^{14}C-labelled taurine activity within 10 minutes, and only 0.5–1.0 μg melittin per ml was accordingly used in the present investigation. Addition of 30 μM BEL or 40 μM AACOCF$_3$ reduces the maximal rate constant for the melittin-induced taurine efflux significantly by 90% and 60% respectively (Table 1), indicating that the melittin-induced activation of taurine efflux from NIH3T3 cells involves iPLA$_2$ as well as cPLA$_2$ activity. The melittin-induced taurine efflux is reduced by BHT and potentiated by H_2O_2 (Fig. 4*B*), and it is estimated that the maximal rate constant, obtained by melittin stimulation, is significantly reduced by 30% in the presence of 0.5 mM BHT and stimulated about 2-fold by 2 mM H_2O_2 (Table 1). Exposing the NIH3T3 cells to 20 mM NAC or 25 μM DI reduces the melittin-induced efflux to 30% (Table 1) and 60 ± 7% ($n = 4$ sets of paired experiments) of the control value, re-

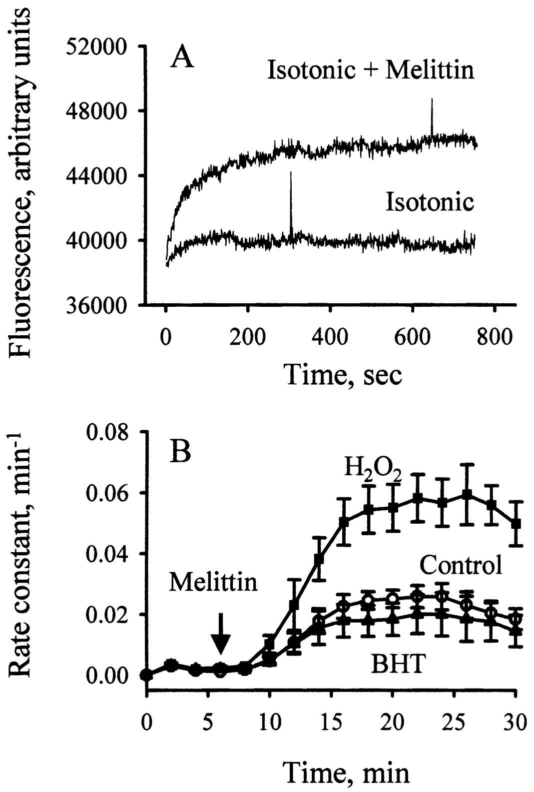

Fig. 4. Effect of melittin on ROS production and taurine efflux from NIH3T3 mouse fibroblasts. (*A*) Cells, grown on coverslips, were loaded with carboxy-H_2DCFDA. The cells were washed and at time zero transferred to isotonic NaCl medium with or without melittin (1 µg/ml). ROS production was followed with time as outlined in Material and Methods. The traces are representative of 3 sets of experiments. (*B*) Cells were loaded with [^{14}C]-taurine for 2 hr in DMEM. The efflux experiments were performed in isotonic NaCl medium, with melittin (0.5 µg/ml) being included in the efflux medium 6 min after initiation of the efflux, as indicated by the arrow. H_2O_2 (2 mM, *squares*) and BHT (0.5 mM, *triangles*) were present in the experimental efflux media throughout the whole efflux experiment. Control cells (*circles*) were not exposed to H_2O_2 or BHT. Mean values ± SEM for the rate constant for taurine efflux were estimated and plotted as a function of time. The curves in *B* represent data from 6 (control), 4 (BHT) and 7 (H_2O_2) sets of experiments.

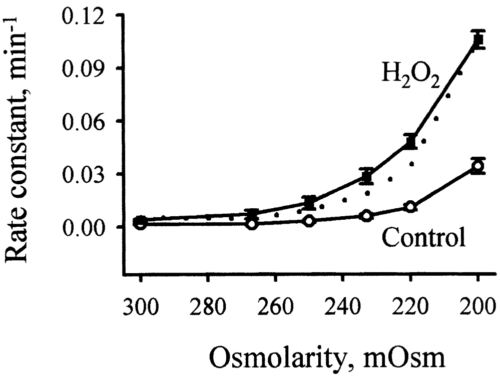

Fig. 5. Effect of H_2O_2 on the set point for activation of the volume-sensitive taurine efflux in NIH3T3 cells. Cells were loaded with [^{14}C]-taurine for 2 hr in DMEM and the efflux experiments were performed in NaCl medium with a shift in osmolarity from 300 mOsm to values in the range 300 to 200 mOsm. The maximal rate constant for the swelling-induced taurine efflux was estimated and plotted versus the extracellular osmolarity. H_2O_2 (2 mM, *filled squares*) was included in the efflux media throughout the whole efflux experiment. Control cells (*empty circles*) were not exposed to H_2O_2. Maximal rate constants are given as mean values ± SEM, and represent 3 (H_2O_2) and 7 (control) independent sets of experiments. The dotted line represents the transformed control curve obtained by multiplication of the data for control cells with the ratio between the maximal rate constants after exposure to hypotonic medium (200 mOsm) in the presence and absence of H_2O_2.

spectively. This is taken to indicate that the NAD(P)H oxidase is also involved in the melittin-induced ROS production and that ROS regulate the subsequent taurine release in NIH3T3 cells. From Table 1 it is also seen that addition of 10 µM ETH 615-139, 50 µM arachidonic acid and 100 µM DIDS reduces the maximal rate constant for the melittin-induced taurine efflux by 85% or more. Furthermore, mobilizing intracellular Ca^{2+} by addition of ATP at the time of hypotonic exposure or addition of melittin leads to a significant potentiation of the induced taurine efflux (Table 1). Taking the pharmacological data in Table 1 into consideration it is suggested that swelling- and melittin-induced cell signalling and taurine release NIH3T3 cells involve elements in common.

ROS DOES NOT AFFECT THE SET POINT FOR ACTIVATION OF THE VOLUME-SENSITIVE TAURINE EFFLUX PATHWAY

Figure 5 demonstrates the maximal rate constant for taurine efflux from NIH3T3 cells, obtained by exposure to isotonic/hypotonic media in the range of 300 mOsm to 200 mOsm, plotted versus the extracellular osmolarity for control cells and for cells exposed to 2 mM H_2O_2. Comparison of the maximal rate constant, obtained by hypotonic exposure, with the rate constant for taurine efflux under isotonic conditions indicated that the volume-sensitive taurine efflux pathway in the presence of H_2O_2 is activated at the same extracellular osmolarity (250 mOsm) as in control cells. Furthermore, multiplication of the data points for the control curve in Fig. 5 with the ratio of the maximal rate constants for H_2O_2 and control cells obtained at 200 mOsm gives a curve that almost superimposes the H_2O_2 curve (Fig. 5, *dotted line*). Thus, the reduction in the extracellular tonicity required for activation of the volume-sensitive taurine efflux pathway is not affected by exogenous ROS. Assuming that NIH3T3 cells swell like perfect osmometers and have a cell water content about 0.76 ml/g wet weight, it is estimated that a 15% cell swelling is required for activation of the taurine-releasing system.

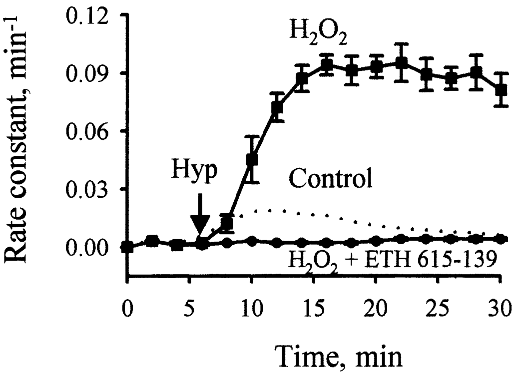

Fig. 6. Effect of 5-LO inhibition on the H_2O_2-induced potentiation of the swelling-induced taurine efflux from NIH3T3 mouse fibroblasts. Cells were loaded with [^{14}C]-taurine for 2 hr in DMEM and the efflux experiments were performed in NaCl medium with a shift in osmolarity from 300 to 200 mOsm, as indicated by the arrow. H_2O_2 (2 mM, *squares*) and H_2O_2 plus ETH 615-139 (10 µM, *circles*) were present throughout the whole efflux experiment. Rate constants for taurine efflux are shown as mean values ± SEM of 4 sets of paired experiments. The dotted line represents the data for control cells from Fig. 3*A*.

From Fig. 6 it is seen that inhibition of 5-LO (10 µM ETH 615-139) essentially abolishes the swelling-induced taurine efflux even in the presence of H_2O_2. As H_2O_2 does not change the volume set-point for activation of the volume-sensitive taurine efflux (Fig. 5) and as H_2O_2 requires 5-LO activity to exert its effect (Fig. 6), it is suggested that an increase in ROS is not the initial signal for activation of the volume-sensitive taurine efflux, but that ROS interfere with the signalling-cascade involved in the regulation of the pathway in NIH3T3 cells.

ROS Interfere with Tyrosine Phosphorylation

Addition of the protein tyrosine phosphatase inhibitor vanadate and the protein tyrosine kinase inhibitor genistein has recently been reported to potentiate and inhibit, respectively, the swelling-induced taurine efflux in NIH3T3 cells (Pedersen et al., 2002). This is illustrated in Fig. 7 and quantified in Table 2. It is estimated that 50 µM vanadate increases the maximal rate constant for taurine efflux in hypotonic (200 mOsm) NaCl medium and KCI medium about 4-fold (Table 2). The EC_{50} value for vanadate-induced potentiation of the swelling-induced taurine efflux was estimated at 73 ± 16 µM (*n* = 3, vanadate in the range of 5–200 µM). Vanadate (50 µM) also increased the maximal rate constant for the melittin-induced taurine efflux under isotonic conditions 2.7 ± 0.3-fold (*n* = 10 paired experiments). The potentiating effects of H_2O_2 and vanadate on the swelling-induced taurine efflux are synergistic, i.e., the rate constant 2 min after hypotonic exposure (200 mOsm) was in 3 sets of experiments estimated at 0.024 ± 0.003 min^{-1} (2 mM H_2O_2), 0.022 ± 0.004 min^{-1} (50 µM vanadate) and 0.0727 ± 0.0117 min^{-1} (vanadate plus H_2O_2). The equivalent rate constants 4 min after hypotonic exposure were 0.071 ± 0.009 min^{-1}, 0.060 ± 0.008 min^{-1} and 0.163 ± 0.007 min^{-1}. It therefore seems reasonable to suggest that ROS inhibit a protein phosphatase, leading to an increased tyrosine phosphorylation of a regulatory protein and subsequent potentiation of the swelling-induced taurine release. Exposing the NIH3T3 cells to the protein tyrosine kinase inhibitors genistein (100 µM) or PD153035 (10 nM) reduces the maximal rate constant for the swelling-induced taurine efflux from NIH3T3 cells by

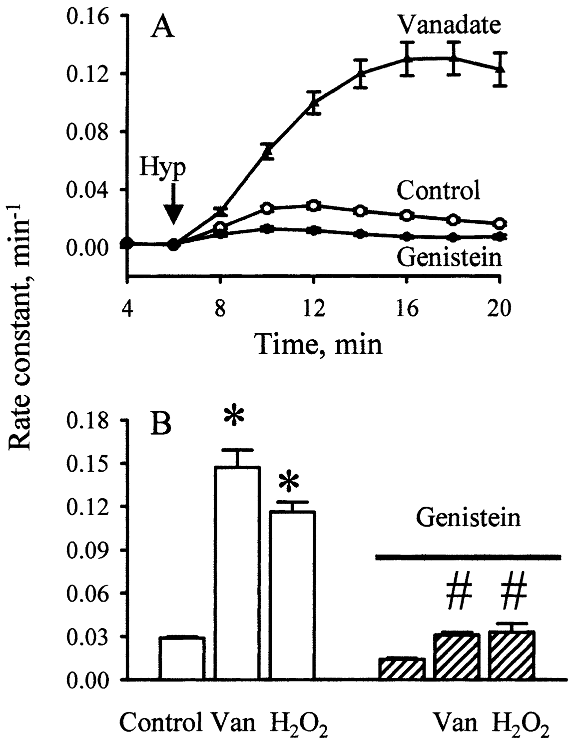

Fig. 7. Effect of Genistein and NaVanadate on the swelling-induced taurine release from NIH3T3 mouse fibroblasts. Cells were loaded with [^{14}C]-taurine for 2 hr in DMEM and the efflux experiments were performed in NaCl medium with a shift in osmolarity from 300 to 200 mOsm 6 min after initiation of the efflux experiment. The rate constant for taurine efflux was estimated and plotted as a function of time. (*A*) Genistein (100 µM, *filled circles*) and NaVanadate (50 µM, *filled triangles*) were present in the experimental media throughout the whole efflux experiment. Control cells (*empty circles*) were only exposed to hypotonicity. The arrow indicates the shift to hypotonicity. Curves are representative of 17 (control), 20 (vanadate) and 12 (genistein) independent sets of experiments. (*B*) The maximal rate constant following hypotonic exposure was estimated in control cells (*n* = 127), cells exposed to vanadate (50 µM, *n* = 20), cells exposed to H_2O_2 (2 mM, *n* = 19), cells exposed to genistein (100 µM, *n* = 13) and cells exposed to genistein plus H_2O_2 (*n* = 4) or genistein plus vanadate (*n* = 3). Values are given as means ± SEM. When not visible the SEM value is hidden in the bar. *Indicates significantly larger than hypotonic control with no additives. $^{\#}$Indicates significantly lower than the equivalent with no genistein added.

Table 2. Effect of protein tyrosine/phosphatase inhibition on the swelling-induced taurine efflux from NIH3T3 mouse fibroblasts

Inhibitor	Taurine efflux maximal rate constant[1]			
	NaCl medium	*n*	KCl medium	*n*
Control	1		1	
NaVanadate	4.17 ± 0.32*	20	3.58 ± 0.37*	8
Genistein	0.44 ± 0.05*	13	0.38 ± 0.03*	4
PD153035	—		0.80 ± 0.01*	4

[1] The maximal rate constant obtained by cell swelling in the presence of inhibitors is given relative to the respective control. Cells were loaded with [^{14}C]-taurine for 2 hr in DMEM. The efflux experiments were performed in either isotonic NaCl or isotonic KCl medium with a shift to hypotonicity (200 mOsm) 6 to 8 min after initiation of the efflux experiment as outlined in Fig. 1. The protein tyrosine phosphatase inhibitor NaVanadate (50 µM) and the protein tyrosine kinase inhibitor genistein (100 µM) and PD153035 (100 nM) were present in the efflux media throughout the efflux experiment, *n* is the number of paired experiments. Asterix indicates data significantly different from the hypotonic control value.

60% and 20%, respectively (Table 2). It is noted that some experiments presented in Table 2 were performed in hypotonic KCl medium in order to prevent the reduction in the cell volume following the initial cell swelling and to prolong the time period where the taurine transport pathway is maximally active (Kirk & Kirk, 1993). As PD153035 inhibits protein tyrosine kinase activity coupled to the epidermal growth factor (EGF) receptor, it is suggested that swelling-induced taurine efflux is regulated by protein tyrosine kinases as well as by tyrosine phosphatases, and that the EGF receptor protein tyrosine kinase could be involved. The latter notion is supported by the observation that addition of EGF potentiates the rate constant for the swelling-induced taurine efflux from NIH3T3 cells 1.09 ± 0.06-fold (50 ng/ml, *n* = 8 paired sets of experiments) and 1.07 ± 0.03 fold-(100 ng/ml, *n* = 4 paired sets of experiments). From Fig 7*B* it is seen that the potentiating effect of H_2O_2 and vanadate on the swelling-induced taurine efflux is impaired in the presence of genistein, indicating that protein tyrosine kinase activity is required in order to see the effect of ROS. Exposing the NIH3T3 cells to 10 or 50 nM PP2, which inhibits the activation of the focal adhesion kinase (FAK), increases the maximal rate constant for the swelling-induced taurine efflux significantly by 1.7 ± 0.1-fold and 2.3 ± 0.1-fold (*n* = 4 paired sets of experiments), respectively. PP3, the negative control for PP2, had no effect on the swellings-induced taurine efflux. This indicates that FAK is also involved in the regulation of swelling-induced taurine efflux in NIH3T3 cells.

Discussion

SWELLING-INDUCED ACTIVATION OF iPLA$_2$

Mammalian cells generally contain more than one type of PLA$_2$ and the physiological consequences following PLA$_2$ activation depend on the cell type, stimuli, the isoform, substrate specificity, and the subcellular localization (substrate availability) of the PLA$_2$ activated. The PLA$_2$ family includes the ubiquitously expressed, cytosolic, Ca^{2+}-dependent PLA$_2$ (cPLA$_{2\alpha}$, group IVA, 85 kDa), the secretory, Ca^{2+}-dependent PLA$_2$ (sPLA$_2$; group IB, IIA-F, V, X; 14–18 kDa), and the cellular, Ca^{2+}-independent, PLA$_2$ (iPLA$_2$; group VIA, 85–89 kDa)(Balsinde et al., 1999). The cPLA$_2$, which preferentially hydrolyzes arachidonic acid from the phospholipids, is involved in the activation of the volume-sensitive osmolyte channels in Ehrlich cells (Thoroed et al., 1997; Pedersen et al., 2000), as well as in CHP-100 neuroblastoma cells (Basavappa et al., 1998). To gain full catalytic activity, cPLA$_{2\alpha}$ requires (i) phosphorylation at Ser^{505} by members of the mitogen-activated protein kinase family (Gijon & Leslie, 1999; Winstead, Balsinde & Dennis, 2000) and (ii) submicromolar Ca^{2+} concentration for translocation from the cytosol to the perinuclear membrane where it docks at the intermediate filament component (vimentin) and gets access to the perinuclear phospholipids as well as the eicosanoid-generating enzymes (5-LO, COX-1, COX-2) (Murakami et al., 1998, 2000). It is noted that the translocation of cPLA$_{2\alpha}$ in, e.g., Ehrlich cells is not prevented by inhibitors of protein kinase C, mitogen activated protein (MAP) kinases or protein tyrosine kinases (Pedersen et al., 2000), and that no Ca^{2+} signalling is recorded in suspension of Ehrlich cells following hypotonic exposure (Jørgensen et al., 1997). Thus, phosphorylation of cPLA$_{2\alpha}$ by these protein kinases is not essential for the translocation and activation of cPLA$_{2\alpha}$, and the prevailing cellular Ca^{2+} concentration is apparently sufficient for binding of cPLA$_{2\alpha}$ to the nucleus in Ehrlich cells. The sPLA$_2$ contains a secretion signal peptide and sPLA$_2$ that belong to the heparin-binding type of sPLA$_2$ (group IIA, IID, V) bind to cell surface heparan sulfate proteoglycan and mediate stimulus-dependent release of arachidonic acid (Murakami et al., 2000). sPLA$_2$ (group X) avidly hydrolyze phosphatidyl choline in the outer leaflet of the plasma membrane in the absence of costimulators and constitute a generous supplier of exogenous lysophosphatidyl choline (LPC) (Murakami et al., 2000; Winstead et al., 2000). In this context it is noted that LPC, when added exogenously, induces release of taurine from various cell types including NIH3T3 cells (Lambert & Falktoft, 2000, 2001; Lambert et al., 2001) as well as ROS production in NIH3T3 cells (*data not shown*). The volume regulatory response in Ehrlich cells, which follows osmotic cell swelling, is besides being inhibited by the $cPLA_2$ inhibitor $AACOCF_3$ (Thoroed et al., 1997) also inhibited by RO 31-4639 (Lambert & Hoffmann, 1991). As RO 31-4639 was originally designed to block pancreatic $sPLA_2$ (Henderson, Chapell & Jones, 1989), its inhibitory effect could well reflect that $sPLA_2$ together with $cPLA_2$ participates in the swelling-induced osmolyte release from Ehrlich cells. The $iPLA_2$ is ubiquitously expressed, exhibits lysophospholipase as well as PLA_2 activity, and has been assigned a role in remodeling of phospholipids and distribution of arachidonic acid in subcellular compartments (Balsinde et al., 1999). The active form of $iPLA_2$ (group VIA) is a tetramer and it has been suggested that ankyrin repeats (eight per monomer) are responsible for the oligomerization of the $iPLA_2$ monomers required for activation of PLA_2 activity (Winstead et al., 2000). $iPLA_2$ (group VIA) is involved in leukotriene synthesis in granulocytes (Larsson Forsell et al., 1998) and prostaglandin synthesis in HEK293 cells transfected with $iPLA_2$ cDNA from hamster (Murakami et al., 1999). Melittin is often used as an activator of $sPLA_2$ and the data in Fig. 4 indicate that melittin, when added to NIH3T3-cells under isotonic conditions, induces ROS production as well as taurine efflux. These effects of melittin involve activation of cellular-signalling and taurine-releasing pathways that demonstrate close pharmacological resemblance to the pathways activated by osmotic cell swelling (Table 1). It is noted that 5 μM melittin, i.e., at a concentration that is ten times higher than the concentrations used in the present paper, previously has been reported to activate lipases other than $sPLA_2$ and to result in release of saturated, monounsaturated as well as polyunsaturated free fatty acids, which subsequently could result in extensive alteration of lipid composition in the cellular membranes and initiation of diverse physiological effects (Lee et al., 2001). As the melittin-induced taurine efflux from NIH3T3 cells is significantly reduced in the presence of the $cPLA_2$ inhibitor AACOCF3 and the $iPLA_2$ inhibitor BEL (Table 1), it seems reasonable to assume that the melittin-induced activation of taurine efflux in the present investigation involves stimulation of $sPLA_2$, $cPLA_2$ and $iPLA_2$. The swelling-induced taurine release from NIH3T3 is unaffected by AACOCF3 (Table 1), slightly inhibited by the $sPLA_2$ inhibitor manoalide (1 μM reduced the swelling-induced taurine efflux by 20%, $n = 2$) and significantly inhibited by BEL (Fig. 1*A*, Table 1). According to a recently published model, it appears that the active site of $iPLA_2$ in the absence of calmodulin interacts with a calmodulin-binding domain within $iPLA_2$ and that binding of calmodulin results in loss of enzymatic activity (Jenkins et al., 2001). Exposure of the NIH3T3 cells to hypotonic medium in the presence of the calmodulin antagonist W7 potentiates the subsequent taurine efflux (Fig. 1*A*). Thus, iPLA is required for swelling-induced taurine release from the NIH3T3 cells. The volume-sensitive, $iPLA_2$-activating event in NIH3T3 cells awaits identification.

ORIGIN OF ROS—ROLE OF THE NAD(P)H OXIDASE

ROS (hydrogen peroxide, superoxide anions, hydroxyl radicals, peroxynitrite) have been assigned a role as mediators of normal and pathological signal transduction (Hensley et al., 2000; Thannickal & Fanburg, 2000; Finkel, 2001). ROS are normally produced as by-products of general cellular metabolism and by a membrane-associated NAD(P)H oxidase system in most cell types (Thannickal & Fanburg, 2000). However, even though cells normally have a very efficient antioxidant defense (superoxide dismutase, catalase, gluthation-dependent peroxidase), the cellular concentration of ROS increases in NIH3T3 cells following hypotonic exposure (Fig. 2) and following addition of melittin (Fig. 4*A*). The swelling-induced ROS production in NIH3T3 cells is reduced in the presence of BEL (Fig. 2*D*), and as the PLA_2 products arachidonic acid and LPC are both reported to generate ROS in a process that requires NAD(P)H-oxidase activity (Kugiyama et al., 1999; Hensley et al., 2000; Yamakawa et al., 2002) it seems reasonable to suggest that the ROS-producing step in NIH3T3 cells following osmotic exposure is downstream to the $iPLA_2$ activation and most probably involves arachidonic acid and/or LPC as well as the NAD(P)H oxidase complex. Taurine release from NIH3T3 cells, following osmotic cell swelling or melittin stimulation, is potentiated by H_2O_2 but reduced in the presence of the antioxidants BHT and NAC, as well as in the presence of the NAD(P)H oxidase inhibitor DI (Figs. 3 and 4; Table 1 and Results). It is noted that H_2O_2 was recently reported to induce a concentration- and time-dependent release of arachidonic acid from U937 cells by a mechanism that involves $iPLA_2$ (not $cPLA_{2\alpha}$) activation, oxidation of membrane lipids and, consequently, increased substrate accessibility for $iPLA_2$ (Balboa & Balsinde, 2002). However, taurine release from NIH3T3 cells is not affected by H_2O_2 when added to the cells under isotonic conditions (Results), i.e., cell swelling is required in order to get an effect of ROS on the taurine release in NIH3T3 cells. The observation that the closing/inactivation of the swelling-induced taurine efflux is delayed in the presence of H_2O_2 (Fig. 3*A*) but accelerated in the presence of DI (Fig. 3*C*), indicates that ROS most probably increase the open-probability of the volume-sensitive taurine efflux pathway in NIH3T3 cells. Protein kinase C (PKC) promotes phosphorylation of p47^{phox}, a component of the NAD(P)H oxidase complex, and subsequently activation of the complex (Fontayne et al., 2002). Swelling-induced taurine efflux from NIH3T3 cells is also potentiated following stimulation of PKC, but the PKC-mediated effect is impaired in the presence of DI (Lambert, 2003). Furthermore, Rac2 is also essential for assembly of the NAD(P)H oxidase complex and expression of constitutively active form of Rac1, a homolog to Rac2, increases the intracellular ROS level in fibroblasts (Finkel, 2001), whereas expression of constitutively activated forms of Rac1 (Rac1V12) in fibroblasts more than doubles the volume regulatory response following hypotonic exposure (Pedersen et al., 2002). Taking these observations into consideration, it seems reasonable to suggest that ROS are generated by the NAD(P)H oxidase system in NIH3T3 cells upon cell swelling, and that they potentiate the subsequent swelling-induced taurine release.

ROS and 5-LO Activity

Volume-sensitive taurine efflux pathways in Ehrlich cells (Lambert & Hoffmann, 1993), human fibroblasts (Mastrocola et al., 1993), cerebellar astrocytes (Sanchez-Olea et al., 1995), HeLa cells (Lambert & Sepulveda, 2000), C2C12 myotubes and myotubes derived from primary porcine satellite cells (Lambert et al., 2001) and NIH3T3 cells (Fig. 1) are blocked by multiple 5-LO inhibitors, and the 5-LO has accordingly been assigned a permissive role in the activation of volume-sensitive taurine pathways. Ca^{2+} increases the hydrophobicity of 5-LO and thereby promotes membrane association, whereas the ability of H_2O_2 and hydroperoxides to activate 5-LO involves oxidation of the non-heme iron of the 5-LO from Fe^{2+} to Fe^{3+} (Musser & Kreft, 1992). The potentiating effect of ATP on the swelling- and melittin-induced taurine release (Table 1) could well be secondary to ATP-induced Ca^{2+} mobilization. H_2O_2 does not affect the osmosensitivity, i.e., the degree of cell swelling required for activation of the volume-sensitive taurine efflux pathway (Fig. 5), and the potentiating effect of H_2O_2 on the swelling-induced taurine efflux is only seen when the 5-LO is active (Fig. 6). This is taken to indicate that ROS interfere at a step upstream of the swelling-induced activation of 5-LO or that ROS directly stimulate the 5-LO and subsequently increase oxidation of arachidonic acid, i.e., the availability of second messengers required for downstream activation of the volume-sensitive taurine efflux pathway. Preliminary estimation of LTB_4 production (enzyme immunoassay, Amersham Pharmacia Biotech), as an estimate of 5-LO activity, indicated a 25% increase in LTB_4 synthesis under isotonic conditions following stimulation with H_2O_2 (2 mm, *data not shown*). Although H_2O_2 does not induce taurine release from NIH3T3 cells under isotonic conditions, it is feasible that ROS, produced upon osmotic cell swelling, could stimulate the 5-LO activity and thereby increase the amount of essential second messengers upon cell swelling and subsequently potentiate the taurine efflux. The second messenger in question in NIH3T3 cells has not yet been identified.

ROS Inhibit Protein Tyrosine Phosphatase Activity

The swelling-induced taurine efflux from NIH3T3 cells is potentiated in the presence of the protein tyrosine phosphatase inhibitor vanadate and inhibited in the presence of the protein kinase inhibitor genistein (Fig. 7, Table 2; Pedersen et al., 2002). Similar effects of vanadate and genistein on the volume regulatory response following osmotic exposure have previously been demonstrated for human intestine 407 cells (Tilly et al., 1993). Protein tyrosine phosphatases contain an essential cystein residue in the catalytic site, which forms a thiol-phosphate intermediate during the catalytic process. Vanadate is a phosphate analogue and acts as a competitive inhibitor of the protein tyrosine phosphatase PTP1B (Huyer et al., 1997; Vepa et al., 1999). H_2O_2, on the other hand, oxidizes the cysteine hydroxyl group, which leads to loss of phosphatase activity (Meng Fukada & Tonks, 2002), In vivo stimulation of Rat-1 cells with exogenous H_2O_2 has recently been demonstrated to lead to oxidation of multiple protein tyrosine phosphatases in the molecular range 40–120 kDa (Meng et al., 2002), and as the potentiating effects of H_2O_2 and vanadate on the swelling-induced taurine are synergistic (Results), it seems reasonable to assume that the effect of ROS (H_2O_2) on the swelling-induced taurine efflux from NIH3T3 cells reflects oxidation and subsequent inhibition of protein tyrosine phosphatase (PTP1B) activity. H_2O_2 and vanadate do not induce taurine release from-NIH3T3 cells under isotonic condition (Figs. 3 and 7; Table 1; Pedersen et al., 2002) and their potentiating effect on the swelling-induced taurine release is impaired in the presence of genistein (Fig. 7). Thus, the ROS-mediated effect on taurine efflux from NIH3T3 cells upon cell swelling most probably reflects a shift in protein tyrosine phosphorylation due to a concomitant stimulation of protein tyrosine kinase activity and inhibition of protein tyrosine phosphatase (PTP1B) activity.

Protein tyrosine phosphorylation has previously been associated with activation of volume-sensitive ionic conductances (Davis et al., 2001) in a process that in human intestine 407 cells involves the focal adhesion kinase (FAK, $p125^{FAK}$) (Tilly et al., 1996). FAK is a non-receptor tyrosine kinase that lacks the Src homology domains (SH2, SH3) but contains proline-rich sequences plus several tyrosine residues (Ben Mahdi, Andrieu & Pasquier, 2000). Tyrosine phosphorylation of FAK is stimulated by growth factors (Haimovich et al., 1999), by osmotic exposure (Tilly et al., 1993) and following exposure to tyrosine phosphatase inhibitors and H_2O_2 (Vepa et al., 1999), whereas tyrosine phosphorylation of FAK is attenuated by inhibitors of $iPLA_2$ (Haimovich et al., 1999). The inhibitory effect of BEL on ROS production and taurine release (Figs. 1 and 2) and the potentiating effect of H_2O_2 (Figs. 3, Table 1) on taurine release from NIH3T3 cells could accordingly reflect that FAK in a phosphorylated state favors the open state of the volume-sensitive taurine efflux pathway in NIH3T3 cells. However, inhibition of FAK by PP2 potentiates the swelling-induced taurine efflux from NIH3T3 cells (Results), which could indicate that ROS-mediated protein tyrosine phosphorylation of FAK leads to inhibition of its activity. The exact role of FAK in the volume regulatory process and the identification of the proteins that become more tyrosine-phosphorylated by the ROS-sensitive protein tyrosine kinases/phosphatases await further investigation.

The present data demonstrated that (i) $iPLA_2$, 5-LO, protein tyrosine kinases/phosphatases and ROS generating systems are important elements in the signalling sequence that is activated by osmotic cell swelling and that leads to osmolyte release and subsequently to restoration of the cell volume; (ii) it is possible to activate the volume-sensitive, cellular signalling cascade and taurine efflux pathway under isotonic conditions by addition of the lipase activator melittin, and (iii) ROS-induced potentiation of the swelling-induced taurine efflux involves inactivation of a yet unidentified tyrosine phosphatase.

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