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Cell Swelling Activates Phospholipase A_2 in Ehrlich Ascites Tumor Cells

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Abstract. Ehrlich ascites tumor cells, loaded with ^{3}H-labeled arachidonic acid and ^{14}C-labeled stearic acid for two hours, were washed and transferred to either isotonic or hypotonic media containing BSA to scavenge the labeled fatty acids released from the cells. During the first two minutes of hypo-osmotic exposure the rate of ^{3}H-labeled arachidonic acid release is 3.3 times higher than that observed at normal osmolality. Cell swelling also causes an increase in the production of ^{14}C-stearic acid-labeled lysophosphatidylcholine. This indicates that a phospholipase A_2 is activated by cell swelling in the Ehrlich cells. Within the same time frame there is no swelling-induced increase in ^{14}C-labeled stearic acid release nor in the synthesis of phosphatidyl ^{14}C-butanol in the presence of ^{14}C-butanol. Furthermore, U7312, an inhibitor of phospholipase C, does not affect the swelling induced release of ^{14}C-labeled arachidonic acid. Taken together these results exclude involvement of phospholipase A_1, C and D in the swelling-induced liberation of arachidonic acid. The swelling-induced release of ^{3}H-labeled arachidonic acid from Ehrlich cells as well as the volume regulatory response are inhibited after preincubation with $GDP_\beta S$ or with $AACOCF_3$, an inhibitor of the 85 kDa, cytosolic phospholipase A_2. Based on these results we propose that cell swelling activates a phospholipase A_2—perhaps the cytosolic 85 kDa type—by a partly G-protein coupled process, and that this activation is essential for the subsequent volume regulatory response.

Key words: Arachidonic acid release — Cytosolic phospholipase A_2 — Phospholipase D — G-proteins — Cell volume regulation

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Introduction

A reduction in the osmolality of the extracellular fluid or a net uptake of nutrients make animal cells swell. Due to net efflux of inorganic and/or organic osmoeffectors the cells subsequently shrink and regain their normal cell volume. This process is generally termed regulatory volume decrease (RVD). In some types of cells increased metabolism of organic osmoeffectors contributes to the RVD as well (*see* Law, 1991). The transport systems mediating volume regulatory efflux of osmolytes have been well characterized in a number of cell types. K^+-Cl^--cotransporters, K^+-channels, Cl^--channels and organic osmolyte channels are the most important transport systems, depending on the type of cells (for review *see* e.g., Hoffmann & Dunham, 1995). The knowledge about the signal transduction cascade involved in the activation of the various volume regulatory transport pathways, on the other hand, is relatively scarce. A number of metabolites of the polyunsaturated fatty acid arachidonic acid have been assigned a role in cell volume regulation. The RVD response as well as the concomitant swelling-induced taurine efflux in Ehrlich mouse ascites tumor cells (Lambert & Hoffmann, 1991, 1993), the RVD response in human platelets (Margalit et al., 1993), and the swelling-activated anion channel in endothelial cells (Nilius et al., 1994) are all inhibited by inhibitors of phospholipase A_2. In Ehrlich cells (Lambert, Hoffmann & Christensen, 1987), human platelets (Margalit et al., 1993), human fibroblasts (Mastrocola et al., 1993), rat colonic epithelial cells (Diener & Scharrer, 1993) as well as in erythrocytes from marine fish species (Thoroed & Fugelli, 1994), the factual evidence is accumulating in support of a very important role for lipoxygenase product(s) in the signal transduction cascade. In Ehrlich cells it has been demonstrated that cell swelling is accompanied by an increased synthesis of leukotrienes (leukotriene C_4 (LTC_4) and leukotriene D_4 (LTD_4), Lam-

bert et al., 1987), and that inhibition of 5-lipoxygenase or blockage of the LTD_4 receptor inhibit the RVD response (Lambert et al., 1987; Lambert, 1989; Lambert & Hoffmann, 1991; Jørgensen, Lambert & Hoffmann, 1996; *see* Hoffmann, Simonsen & Lambert, 1993; *see* Lambert, 1994). In human blood platelets the 12-lipoxygenase and hepoxilin mimick the role of the 5-lipoxygenase and LTD_4 in Ehrlich cells (Margalit et al., 1993). In C6 glioma cells organic osmolyte efflux and anion currents are inhibited by lipoxygenase and cytochrom P-450 blockers (*see* Strange, Emma & Jackson, 1996). However, Strange and co-authors (1996) questioned the use of lipoxygenase inhibitors as these seem to have direct effect on the organic osmolyte channel. In the light of this observation it becomes highly relevant to supply inhibitor studies with direct measurements of changes in the precursors/metabolites involved.

The rate-limiting factor in the synthesis of eicosanoids is generally considered to be the availability of arachidonic acid. This implies that cell swelling, caused by reduction of osmolality, should stimulate phospholipases leading to increased hydrolysis of arachidonic acid from membrane phospholipids (*see* Lambert, 1994; Hoffmann & Dunham, 1995). Swelling-induced activation of phospholipase A_2 has previously been proposed in human blood platelets (Margalit et al., 1993). Margalit and Livne (1992), furthermore, have demonstrated that the same signal cascade can be provoked by mechanical stress. A direct demonstration of shear stress-induced activation of cytosolic phospholipase A_2 and a MAP kinase comes from the work on human endothelial cells by Pearce and coworkers (1996). Thus, cell swelling and shear stress seem to result in activation of common signal cascades, starting with release of arachidonic acid. It has, however, never been demonstrated directly that cell swelling is associated with an increased hydrolysis of arachidonic acid from the membrane phospholipids.

Figure 1 summarizes the different pathways, which result in release of arachidonic acid from membrane phospholipids. The most obvious mechanism of arachidonic acid release is catalized by phospholipase A_2, which primarily hydrolyzes phosphatidylcholine and phosphatidylethanolamine (Exton, 1994). Arachidonic acid, however, can also be released following the action of both phospholipase C and phospholipase D, followed by the action of diacyl- and monoacylglycerol lipase (Burgoyne & Morgan, 1990), and the arachidonic acid released secondary to activation of phospholipase D has been demonstrated to be important in the production of eicosanoids (Linn, Wiggan & Gilfillan, 1991; Ishimoto et al., 1994).

The aim of the present study was to demonstrate whether arachidonic acid is actually released from Ehrlich cells during RVD and to investigate the lipase(s) involved. Part of this paper has been presented in a preliminary form (Thoroed et al., 1994; Hoffmann et al., 1995).

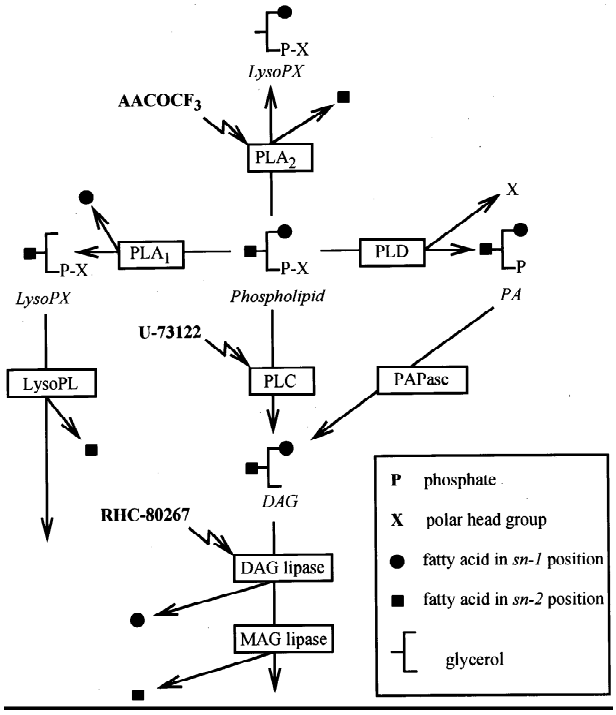

Fig. 1. Potential enzymatic pathways involved in the release of arachidonic acid and stearic acid from phospholipids. Circles represent fatty acids at *sn*-1 position. Squares represent fatty acids in *sn*-2 position. In the present investigation we presume that ^{14}C-labeled stearic acid and ^{3}H-labeled arachidonic acid are predominantly incorporated in *sn*-1 and *sn*-2, respectively. X: the polar head group e.g., choline, ethanolamine, serine and inositol. PLA_1, PLA_2, and PLC and PLD: phospholipase A_1, A_2, C and D, respectively. DAG: diacylglycerol. PA: phosphatidic acid. LysoP: lysophosphatidyl. LysoPL: lysophospholipase, PAPase: phosphatidic acid phosphohydrolase, DAG lipase: diacylglycerol lipase, MAG lipase: monoacylglycerol lipase. Modified from Allen et al., 1992. The site of action of inhibitors used in the present investigation is indicated.

Materials and Methods

Reagents and Incubation Media

BSA (bovine serum albumin-essentially fatty acid free), butylated hydroxy toluene (2,[6]-di-tert-butyl-p-cresol), $GDP_\beta S$ (guanosine 5-O-(2-thiodiphosphate), HEPES (N-(2-hydroxyethyl)piperazine-N^1-(2-ethane sulfonic acid)), MOPS (3-(N-morpholino)propane sulfonic acid), NMDG (N-methyl-D-glucamine), phospholipid standards, PMA (4 β-phorbol 12-myristate 13-acetate) and TES (N-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid) were obtained from Sigma Chemical (St. Louis, MO). $AACOCF_3$ (an arachidonic acid analogue where the COOH-group of arachidonic acid has been replaced by a $COCF_3$-group), RHC-80267 (1,6-bis-(cyclohexyloximino-carbonylamino)-hexane, and U-73122 (1-6-(17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-1H-pyrrole-2,5-dione) were

obtained from BIOMOL Research Lab. (Plymouth Meeting, PA). ^{3}H-arachidonic acid (100 Ci/mmol) and ^{14}C-butanol (1.01 mCi/mmol) were obtained from DuPont NEN (Bad Hamburg, Germany). ^{14}C-cholesterol (52 mCi/mmol) and ^{14}C-stearic acid (51 mCi/mmol) were from Amersham International (Amersham, UK). All other reagents were of analytical grade.

The standard incubation medium contained the following solutes (mM): NaCl, 143.0; KCl, 5.0; $MgSO_4$, 1.0; Na_2HPO_4, 1.0; $CaCl_2$, 1.0; MOPS, 3.3; TES, 3.3 and HEPES, 5.0. NaOH was added to adjust pH at 7.40. The osmolality of the medium was 300 mOsmol/kg as measured with an osmometer (Knauer, Berlin, Germany). Hypotonic medium (157 mOsmol/kg) was obtained by mixing equal volumes of incubation medium and buffered water containing 1 mM $CaCl_2$ and the same concentration of MOPS, TES and HEPES as the standard incubation medium. Hypotonic NMDG medium contained (mM): NaCl, 2.0; NMDG, 69.5; KCl, 2.5; K_2HPO_4, 0.5; $MgSO_4$, 0.5; $CaCl_2$, 1.0; MOPS, 3.3; TES, 3.3 and HEPES, 5.0.

Stock solutions of RHC-80267 (10 mM in dimethyl sulfoxide), $AACOCF_3$ (2.8 mM in ethanol) and U-73122 (10 mM in chloroform) were prepared and kept at room temperature (RHC-80267 and U-73122) or at −20°C ($AACOCF_3$). To prevent exposure of the cells to chloroform and dimethyl sulfoxide, after an aliquot of the stock solution was applied to the incubation bottles, the solvent was evaporated under a stream of nitrogen before addition of cells suspended in medium containing BSA (1.0% (w/v)). The radioactive fatty acids were stored under nitrogen at −20°C.

Cell Culture

Hyperdiploid strain of Ehrlich ascites tumor cells were grown for 8 days in the abdominal cavity of female NMRI (Naval Medical Research Institute) mice. The cell culture was maintained by weekly intraperitoneal transplantation. The mice were fed standard food for rodents and water *ad libitum.* The cells were harvested in incubation medium with added heparin (2.5 IU/ml). The cells were washed twice in heparin-free incubation medium by sedimentation (37°C, 45 sec, 700 × *g*) and resuspension. Cell viability was estimated from the percentage trypan blue positive cells.

Incorporation of Radioactive Fatty Acids in Phospholipids

Following the initial washing procedure 7.2 ml of the cell suspension (cytocrit 0.5%) was transferred to a flask of glass and the cells were loaded with ^{3}H-arachidonic acid (5.8 μCi) and/or ^{14}C-stearic acid (5.8 μCi) for 120 min in a temperature-controlled (37°C), shaking water bath. The radioactive fatty acids were added as tiny droplets scattered around in the cell suspension. The loading period was followed by three washes of the cells in 8-ml incubation medium containing 1.0% (w/v) essentially fat-free bovine serum albumine (BSA). The washing medium contained BSA to scavenge the extracellular fatty acids. Finally the cell pellet was resuspended in BSA-containing incubation medium (1.0% (w/v)) and split in two parts in new glass flasks. The cell suspensions (approximately 0.5% cytocrit) were pre-incubated for at least 20 min.

Measurement of Fatty Acid Release

Ehrlich cells, loaded with ^{3}H-arachidonic acid and washed with medium containing 1.0% BSA as described above, were at time zero diluted with an equal volume of either isotonic standard incubation medium (final osmolality 300 mOsmol/kg) or buffered water (final osmolality 157 mOsmol/kg). The final BSA concentration was 0.5% and the final cytocrit 0.25%. Since BSA binds fatty acids with high affinity (*see* Bojesen & Bojesen, 1994) 0.5% will scavenge all the labeled fatty acids released by the cells. During the following 8 min, aliquots (300 μl) of the cell suspensions were transferred to Eppendorf tubes. The cells were immediately sedimented by centrifugation (45 sec, 1,400 × *g*), and the tubes were put on ice. 200 μl of the supernatant was taken to determine the extracellular concentration of radioactive fatty acids by liquid scintillation spectrometry. Finally, 1.0 ml of the cell suspension was diluted 100 times in incubation medium to determine the concentration of cells (number of cell/ml) using a Coulter counter model Z (Coulter Electronics LTD, Harpenden Herts, UK).

Lipid Extraction and Thin Layer Chromatography

The phospholipids were extracted essentially as described by Lauritzen et al. (1993). About 1.0×10^6 cells, loaded with radioactively labeled fatty acids, were after the three washes transferred to ice-cold tubes of glass. The cells were immediately sedimented (4°C, 45 sec, 700 × *g*) and the supernatant aspirated. 500 μl chloroform containing 0.01% (w/v) of the antioxidant butylated hydroxy toluene was added to the cell pellet. The tubes were sealed and stored at −20°C. When thawed the chloroform and cell pellet were mixed with 500 μl phosphate buffered saline, 900 μl chloroform, and 700 μl methanol. The lower chloroform phase was collected and the aqueous phase was again mixed with 1-ml chloroform. The second chloroform phase was collected, whereafter the combined chloroform extracts were concentrated under a stream of nitrogen and applied to thin layer plates (TLC plates, silica gel 60) (Merck, Darmstadt, Germany). To determine the incorporation of the radioactive fatty acids in various lipid pools, the phospholipids were separated using a mixture of chloroform/methanol/acetic acid/water (60:50:1:4 by volume) as the mobile phase. When determining the incorporation of ^{14}C-stearic acid in lysophospholipids, these lipids were isolated by employing a mobile phase of chloroform/methanol/acetic acid/water (50:30:8:4 by volume). The various radioactive lipid spots on the TLC plates were localized by autoradiography for 6–7 days and identified by comparison with nonradioactive standards visualized by iodine vapor. Finally, the radioactive spots were scraped off the plate and added 200 μl methanol:1 N HCl (150:1 by volume) and 2 ml Ecoscient. The radioactivity was determined by liquid scintillation spectrometry.

Assay for Phospholipase D Activation

Following the initial washing procedure 3.75 ml cell suspension (cytocrit 6–7%) was added an equal volume of either the incubation medium or buffered water containing 1 mM Ca2+, both solutions contained approximately 24 μCi ^{14}C-butanol (final butanol concentration 0.03% (v/v)). A sample (1 ml) of the cell suspension was taken every minute for 6 min to determine the content of phosphatidyl ^{14}C-butanol (PtdBut). The sample was transferred to an Eppendorf tube, and the cells sedimented (45 sec, 1,400 × *g*). The cell pellet was resuspended in 1 ml of ice-cold water, transferred to a tube of glass and added 2 ml of 2:1 (v/v) mixture of chloroform and methanol containing 9.5 μCi ^{14}C-cholesterol. Cholesterol was added as an internal standard. The tubes were sealed and kept at −20°C.

To identify the Ptd^{14}C-But that was formed in the cells unlabelled PtdBut-standard (25 μg) was added to all the tubes, whereafter the lipids were extracted 3 times. The PtdBut-standard was prepared according to Smith et al. (1978): In short, 1 mg/ml solution of 1-stearoyl-2 arachidonoyl phosphatidylcholine dissolved in diethylether

was mixed with an equal volume of a solution of cabbage phospholipase D (62.5 IU/ml) in a mixture of 5% (v/v) butanol, 40 mM $CaCl_2$ and 50 mM acetic acid (pH 5.6). After an overnight incubation the phospholipase D-activity was terminated by reducing the pH to 1–2 with 2N HCl. The phospholipids of this incubation-mixture were extracted with chloroform and separated by thin layer chromatography using the organic phase of ethylacetate/isooctane/acetic acid/water (90:12:21:70 by volume) as the mobile phase (Lauritzen et al., 1994). This standard gave rise to only two spots, namely PtdBut and phosphatidic acid.

The cellular lipids were separated by thin layer chromatography as described above. The radioactive spots were visualized by autoradiography for two weeks. In all lanes only two spots were evident in the autoradiogram. By comparison with unlabeled standards, visualized by iodine vapor, these spots were identified as PtdBut and cholesterol, respectively. Both spots were scraped off the TLC-plates and measured by liquid scintillation. The radioactivity incorporated in PtdBut was normalized according to the counts in the cholesterol spot thereby correcting for a variable recovery.

Measurement of Cell Volume

The cell volume (fl) was estimated by electronic cells sizing using a Coulter Multisizer and a final cell density at about 90,000 cells/ml. The cell volume was calculated as the median of the cell volume distribution curve, and polystyrene latex beads (13.5 μm diameter, KeBo) were used as standards (*see* Hoffman et al., 1984). The initial rate of regulatory volume decrease (fl/min) was calculated as the rate of cell shrinkage between about 1 and 3 min following reduction in osmolality using linear regression analysis.

Statistics

Linear regression analysis was used to calculate the rate of release of ^{3}H-arachidonic acid and ^{14}C-stearic acid (cpm/min · 10^6 cells) and to calculate the synthesis of Ptd^{14}C-But (dpm/min · ml). Data were analyzed by Student's *t*-test for paired observations. Differences with a *P* value of less than 0.05 were considered significant. The values given in the text are mean values ± SEM and ''*n*'' represents the number of independent sets of experiments.

Results

Cell Swelling is Accompanied by a Transient Increase in the Release of ^{3}H-arachidonic Acid

The Table gives the distribution of ^{3}H-arachidonic acid and of ^{14}C-stearic acid in the various membrane phospholipids in the Ehrlich cells at the time when the cells were exposed to hypotonic medium, i.e., after 120 min loading with the labelled fatty acids, 3 washes followed by a 20 min preincubation. ^{3}H-arachidonic acid appears in every group of lipids investigated with the rank of order: phosphatidylcholine > phosphatidylethanolamine > phosphatidylinositol > neutral lipids > phosphatidylserine > free fatty acids > sphingomyelin, and 47% of the cell assiciated ^{3}H-arachidonic acid is incorporated into phosphatidylcholine. ^{14}C-stearic acid is also found to be incorporated in all the investigated groups of lipids with 45% and 30% incorporated in neutral lipids and phosphatidylcholine, respectively.

Table. The distribution of the ^{3}H-arachidonic acid and the ^{14}C-stearic acid incorporated in cellular lipids in Ehrlich cells at the time for reduction in osmolarity

	Radioactivity (% of total cell associated radioactivity)	
	^{3}H-arachidonic acid	^{14}C-stearic acid
Sphingomyelin	0.11 ± 0.06	0.76 ± 0.03
Phosphatidylcholine	47.1 ± 0.4	29.8 ± 0.6
Phosphatidylserine	1.67 ± 0.09	3.7 ± 0.1
Phosphatidylinositol	13.3 ± 0.2	4.3 ± 0.6
Phosphatidylethanolamine	25.2 ± 0.1	14.6 ± 0.9
Free fatty acids	0.8 ± 0.1	2.17 ± 0.08
Neutral lipids	11.8 ± 0.4	44.7 ± 1.3

Ehrlich cells were loaded with ^{3}H-arachidonic acid (5.8 μCi) and ^{14}C-stearic acid (5.8 μCi) for 120 min and the ^{3}H-activity and ^{14}C-activity were estimated in each lipid group as described in Materials and Methods. The activity in each lipid pool is given in percent of the total cell associated radioactivity. Neutral lipids include triglycerides, diacylglycerol, monoacylglycerol, cholesterol esters and ceramide. Values are mean values ± SEM of three independent sets of experiments. The total amount of cell associated ^{3}H and ^{14}C activity was 253837 ± 10384 dpm and 133991 ± 6235 dpm, respectively.

The results in Fig. 2 (panel A) demonstrate that there is a continuous release of ^{3}H-arachidonic acid from the Ehrlich cells (98 ± 6 cpm/min · 10^6 cells, $n = 50$) when the cells are incubated in medium with normal osmolality (300 mOsmol/kg) containing 0.5% (w/v) BSA. Using reversed phase HPLC analysis (Supelco column, C18, 5 μm particle size, elution with methanol) as described in Lambert and Henke (1991), we verified that released label represented ^{3}H-arachidonic acid and not a radioactive metabolite (*data not shown*). Cell swelling, caused by a reduction of the osmolality from 300 mOsmol/kg to 157 mOsmol/kg, resulted in an immediate increase in the release of ^{3}H-arachidonic acid (Fig. 2*A*). During the initial 2 min following reduction in the osmolality the rate of ^{3}H-arachidonic acid release (cpm/min · 10^6 cells) was 3.3 times higher than the rate of ^{3}H-arachidonic acid release at normal osmolality (Fig. 2*B*). The swelling-induced acceleration of the ^{3}H-arachidonic acid release is biphasic, and during the period 3–8 min after the reduction of osmolality the rate of release is only 1.5 times higher than the rate of the release at normal osmolality. It should be noted that the frequency of sampling was higher during the initial 2-min period than during the 3–8 min period (Fig. 2*A*). A high frequency of sampling could theoretically cause a mechanical stimulation of the cells and result in an increased phospholipase A_2 activity (Margalit et al., 1993). If so, a similar stimulation and deacceleration of the ^{3}H-arachidonic acid release should also be seen from the cells at normal osmolality. Figure 2*A* shows that this is

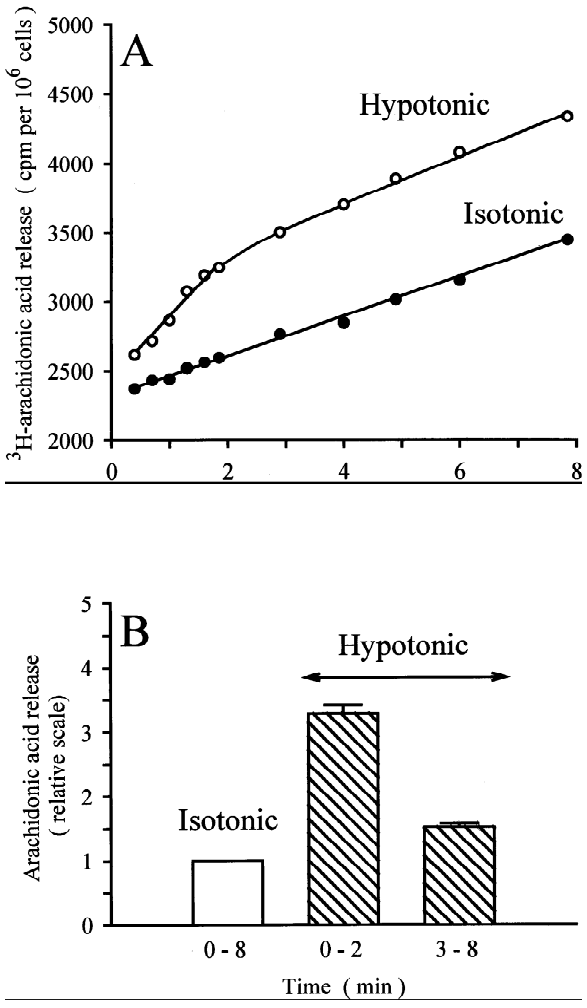

Fig. 2. The effect of reduction of osmolality on the release of ^{3}H-arachidonic acid from Ehrlich cells. (*A*) Ehrlich cells, loaded with ^{3}H-arachidonic acid, were at time zero diluted with an equal volume of either isotonic standard incubation medium (●: final osmolality 300 mOsmol/kg) or buffered water (○: final osmolality 157 mOsmol/kg). Release of arachidonic acid was measured as described in Materials and Methods. The regression lines for the release of ^{3}H-arachidonic acid (cpm/10^6 cells) during the periods 0–2 min ($y = 465x + 2421$; $R = 0.991$; $P < 0.001$) and 3–8 min ($y = 170\,x + 3028$; $R = 0.996$; $P < 0.001$) at 157 mOsmol/kg and for the whole time period at 300 mOsmol/kg ($y = 140\,x + 2325$; $R = 0.998$; $P < 0.001$) are drawn. The figure shows a typical experiment selected from the 50 independent experiments included in *B*. (*B*) The rate of release of ^{3}H-arachidonic acid (cpm/min · 10^6 cells) during different time intervals after reduction of osmolality. The values at 157 mOsmol/kg are given relative to the rate of release at 300 mOsmol/kg. Mean values ± SEM ($n = 50$) are shown. The rate of arachidonic acid release at 300 mOsmol/kg was 97.5 ± 5.8 cpm/min · 10^6 cells. The rate of release during the period 0–2 min at 157 mOsmol/kg was significantly higher than that at 300 mOsmol/kg ($P < 0.001$). The rate of release during the period 3–8 min at 157 mOsmol/kg was significantly lower than the rate of release during the period 0–2 min at 157 mOsmol/kg ($P < 0.001$), but still significantly higher than the rate of release at 300 mOsmol/kg ($P < 0.001$).

not the case. However, to further investigate an eventual role of mechanical stimulation we performed two independent sets of experiments; in one group the frequency of sampling was kept high throughout the whole experimental period whereas the other group was handled according to the standard procedure (*see* legend to Fig. 2*A*). Both groups gave results similar to the ones pictured in Fig. 2*A* (*data not shown*). Thus, hypotonically cell swelling induces a biphasic increase in the rate of ^{3}H-arachidonic acid release from Ehrlich cells.

The increased release of ^{3}H-arachidonic acid during the initial 2 min of RVD only represented 0.15 ± 0.01% ($n = 20$) of the total cell associated ^{3}H-arachidonic acid. We have, therefore, not been able to demonstrate any significant reduction in the content of ^{3}H-arachidonic acid in the phospholipid pools 2 min after the reduction of osmolality (*data not shown*). Thus, from these experiments we are not able to indicate from which pool of phospholipids ^{3}H-arachidonic acid is released after cell swelling.

Phospholipase C and D are Not Involved in the Swelling-induced Increase in the Release of ^{3}H-arachidonic Acid

The increased release of arachidonic acid could be due to increased activity of several phospholipases, working alone or in concert (Fig. 1). To investigate the possible role of the phospholipase C and D multistep pathways in the hypo-osmotically stimulated release of arachidonic acid seen in Fig. 2, we used several alternative approaches. To exclude that phospholipase C is mediating the increased release of ^{3}H-arachidonic acid after a reduction of the osmolality we used U-73122, which is a specific inhibitor of phospholipase C (Bleasdale et al., 1990). The rate of ^{3}H-arachidonic acid release in hypotonic medium following 20 min preincubation with 10 μM U-73122 was 1.0 ± 0.1 ($n = 4$) times the rate of ^{3}H-arachidonic acid release in hypotonic control cells not preexposed to U-731222. 10 μM U-73122 has recently been demonstrated to block the phospholipase C-mediated release of Inositol(1,4,5)P_3 as well as the transient release of Ca^{2+} from intracellular stores after stimulation of Ehrlich cells with LTD_4 and bradykinin (Pedersen et al., 1997).

The release of arachidonic acid via activation of phospholipase D occurs by a multistep pathway involving the action of phosphatidic acid phosphohydrolase followed by diacylglycerol lipase and monoacylglycerol lipase (Fig. 1). The last two steps of this pathway are also involved when arachidonic acid is released from diacylglycerol produced after activation of phospholipase C (Fig. 1; Burgoyne et al., 1990; Allen et al., 1992). Diacylglycerol in general contains a saturated fatty acid in the *sn*-1 position and an unsaturated fatty acid in the

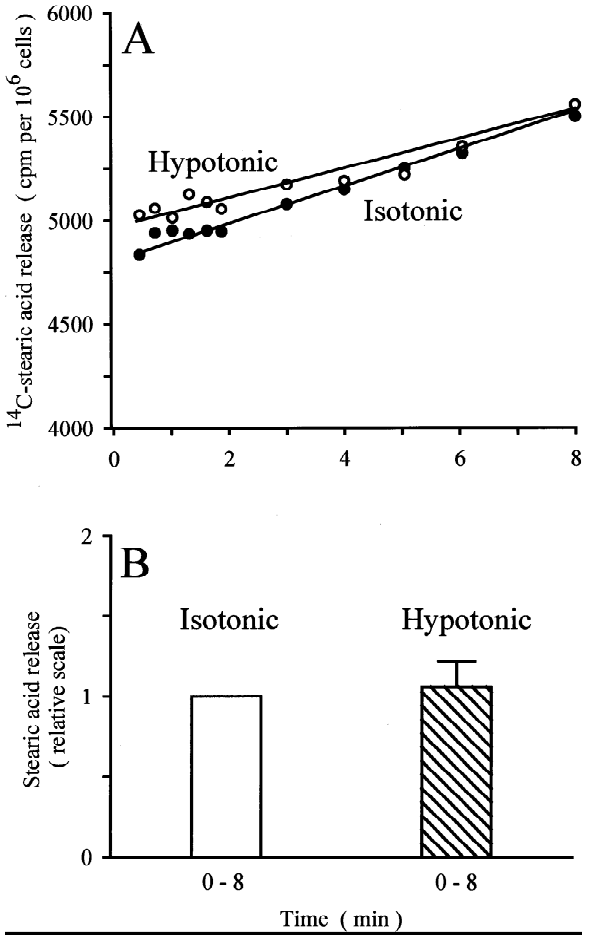

Fig. 3. Effect of cell swelling on the rate of ^{14}C-stearic acid release from Ehrlich cells. (*A*) Ehrlich cells, loaded with ^{14}C-stearic acid, were at time zero diluted with an equal volume of either isotonic standard incubation medium (●: final osmolality 300 mOsmol/kg) or buffered water (○: final osmolality 157 mOsmol/kg). Release of stearic acid was measured as described in Materials and Methods. The rate of ^{14}C-stearic acid release in isotonic media was estimated at 71 ± 20 cpm/min · 10^6 cells (n = 4). The figure shows a typical experiment selected from the 4 experiments included in *B*. (*B*) Values in hypotonic medium are given relative to values in isotonic medium ± SEM (n = 4). The effect of cell swelling on stearic acid release was not significant (P > 0.2). As a control the rate of ^{3}H-arachidonic acid release was measured simultaneously with the release of ^{14}C stearic acid. Given relative to the release under isotonic conditions, this rate was 2.7 ± 0.3 and 1.39 ± 0.07 in the periods 0–2 min and 3–8 min, respectively. The transient stimulation of arachidonic acid is quantitatively similar to the data presented in Fig. 2.

sn-2 position (Allen et al., 1992). Thus, in the described multistep pathway a saturated fatty acid is released from the *sn*-1 position prior to the liberation of arachidonic acid from the *sn*-2 position (*see* Fig. 1). Therefore, we studied the effect of reduction of osmolality on the simultaneous release of ^{14}C-stearic acid and ^{3}H-arachidonic acid. The rate of ^{14}C-stearic acid release was unaltered throughout the 8-min incubation period in hypotonic medium (*see* Fig. 3*A*), whereas the concomitant ^{3}H-arachidonic acid release showed the same variation as shown in Fig. 2*A* (*see* legend to Fig. 3). Thus, in contrast to the release of ^{3}H-arachidonic acid, reduction of the osmolality does not stimulate the release of ^{14}C-stearic acid (Fig. 3*B*). Apart from excluding the involvement of the phospholipase C and D multistep pathways in the ^{3}H-arachidonic acid release the data in Fig. 3 also exclude that phospholipase A_1 and lysophospholipase participate in the observed release of arachidonic acid. Since hydrolysis of diacylglycerol by diacylglycerol lipase precedes the monoacylglycerol lipase-catalyzed liberation of arachidonic acid, we, moreover, investigated the effect of the diacylglycerol lipase inhibitor RHC-80267 (Sutherland & Amin, 1982) on the release of ^{3}H-arachidonic acid. The rate of ^{3}H-arachidonic acid release in hypotonic medium following 30-min preincubation with 30 μM RHC-80267 was 0.86 ± 0.08 (n = 5) times the rate of ^{3}H-arachidonic acid release in hypotonic control cells not preexposed to RHC-80267. The effect of RHC 80267 was not significant. Although we had no positive control for the activity of the inhibitor, 30 μm RHC-80267 is a high concentration, i.e., 5 times higher than IC_{50} (4 μM) given by BIOMOL Research Lab. and thus likely to be sufficient to inhibit the diacylglycerol lipase.

To further demonstrate that phospholipase D is not activated by reduction of osmolality, we also measured phospholipase D activity directly by a specific phospholipase D assay. In the presence of a primary alcohol phospholipase D catalyzes a unique transphosphatidylation leading to the production of phosphatidylalcohol, which is normally not present among cell phospholipids (Alling et al., 1984; Randall et al., 1990). When Ehrlich cells at normal osmolality were exposed to medium added ^{14}C-butanol there was a slow, linear synthesis of phosphatidyl-^{14}C-butanol (0.037 ± 0.0013 Bq/min · ml, n = 3). The rate of synthesis was not significantly affected by a reduction of osmolality from 300 to 157 mOsmol/kg (release was 1.4 ± 0.8 compared to isotonic control, n = 3). The phorbol ester phorbolic myristic acid (PMA), which stimulates phospholipase D in various cell types (Exton, 1994), was included as a positive control of the method. Stimulation of Ehrlich cells with 200 nM PMA at normal osmolality resulted in a 3.0 ± 0.4 (n = 3) fold increase in the formation of phosphatidyl-^{14}C-butanol compared to the non-stimulated cells. Thus, phospholipase D seems not to be activated following hypo-osmotic swelling of the cells.

Taken together these data exclude the participation of the phospholipases A_1, C, and D in the swelling-induced increase in ^{3}H-arachidonic acid release. It should be noted that although the pool of free ^{14}C-stearic acid is larger than that of ^{3}H-arachidonic acid (Table) cell swelling results in an increased release of ^{3}H-

arachidonic acid (Fig. 2) and not of ^{14}C-stearic acid (Fig. 3), indicating that arachidonic acid is most probably not released from the cellular pool of free fatty acid. This is supported by the fact that the steady-state permeability of arachidonic acid in phospholipid bilayers is extremely high at 37°C (Zhang et al., 1996), whereas the measured release is relatively slow (Fig. 2). Furthermore, the free fatty acid pool of ^{3}H-arachidonic acid is far from large enough to account for the observed linear release. Thus, another pool of arachidonic acid must contribute to the release of free arachidonic acid, and we believe that the rate-limiting step of the overall release must be the liberation from this pool.

Phospholipase A_2 is Involved in the Swelling-induced Increase in the Release of ^{3}H-arachidonic Acid

The results presented so far leave a key role to phospholipase A_2. To support the presumed role of phospholipase A_2 in the swelling-induced release of ^{3}H-arachidonic acid we investigated the effect of reduction of osmolality on the production of ^{14}C-lysophospholipids in cells loaded with ^{14}C-stearic acid. As stearic acid predominantly is located in the *sn*-1 position of phospholipids, lysophospholipids containing ^{14}C-stearic acid are primarily produced by cleavage of the *sn*-2 fatty acid by phospholipase A_2 (Fig. 1). During the initial 145 sec following dilution with isotonic solution or buffered water the ^{14}C-activity of lysophosphatidylcholine, in the percentage of the total cell associated radioactivity, increased from 0.13 ± 0.02% to 0.14 ± 0.02 (isotonic medium) and from 0.14 ± 0.02 to 0.19 ± 0.03 (hypotonic medium), i.e., with 0.006 ± 0.001%-point and 0.044 ± 0.007%-point, respectively (Fig. 4). Thus, cell swelling causes a significant increase in the production of ^{14}C-lysophosphatidylcholine along with the ^{3}H-arachidonic acid release, signifying that a phospholipase A_2 is activated.

Inhibition of a Cytosolic Phospholipase A_2 Reduces the Swelling-induced Increase in the Release of ^{3}H-arachidonic Acid as well as the RVD Response

Several types of phospholipase A_2 have been identified and characterized with respect to Ca^{2+} dependency (*see* Dennis, 1994). Since the Ehrlich cells are able to volume regulate in Ca^{2+}-free medium (Hoffmann et al., 1984) it is unlikely that any of the secreted Ca^{2+}-dependent phospholipase A_2 should be involved in the volume regulatory response in the Ehrlich cells. Consequently, we focused on the possible role of a cytosolic phospholipase A_2 and exposed the Ehrlich cells to $AACOCF_3$, an arachidonic acid analogue which is

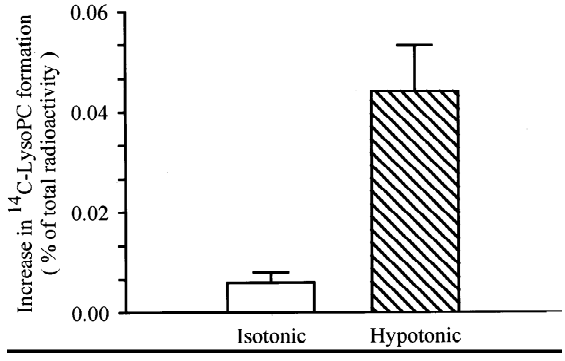

Fig. 4. The effect of reduction of osmolality on the formation of ^{14}C-stearic acid-labeled lysophosphatidyl choline (^{14}C-lysoPC). Ehrlich cells were loaded with ^{14}C-stearic acid as described in Materials and Methods, and the cells were at time zero diluted with either isotonic standard medium or buffered water. The ^{14}C-stearic acid activity in the lysoPC spot was estimated in % of the total cell associated radioactivity. The ^{14}C-stearic acid activity in the stearyl-glycerophosphoryl choline (lysoPC) spot was estimated at time zero and at 145 sec following isotonic and hypotonic exposure. The initial values were 1073 ± 109 dpm (isotonic medium) and 1245 ± 113 dpm (hypotonic medium), which represented 0.13 ± 0.02% and 0.14 ± 0.02% of the total cell associated radioactivity, respectively. The increase in ^{14}C-lysoPC formation is given as the increase in the ^{14}C-stearic acid activity (measured as % of the total cell associated radioactivity) in the lysoPC spot during the 145-sec exposure. Values are ±SEM. ($n = 3$). The formation of ^{14}C-lysoPC was significantly higher after reduction of the osmolality as compared to the formation in cells kept under isotonic conditions ($P = 0.035$).

claimed to be a selective, membrane permeable, slow-acting inhibitor of the 85 kDa cytosolic phospholipase A_2 (Street et al., 1993), which acts by a tight binding to the active site of the phospholipase (Trimblee et al., 1993; *see* Clark et al., 1995). Pre-incubating the Ehrlich cells (cytocrit 0.5%) for 155–160 min with 2 μM $AACOCF_3$ reduced the swelling-induced release of ^{3}H-arachidonic acid by 54% (Fig. 5, panel A). Increasing the concentration from 2 μM to 5 μM improved the inhibitory effect of $AACOCF_3$, although not significantly. The release of ^{3}H-arachidonic acid in the presence of 5 μM $AACOCF_3$ was in 7 paired experiments 0.87 ± 0.09 of the release seen in the presence of 2 μM $AACOCF_3$ ($P = 0.08$). There was no effect of $AACOCF_3$ on the arachidonic acid release under isotonic conditions (*see* legend to Fig. 5*A*).

We also examined whether pre-exposure to $AACOCF_3$ reduced the ability of Ehrlich cells to regulate their volume following cell swelling. Figure 5 (*B*) shows that pre-incubation of the Ehrlich cells (cytocrit 1.0%) for 45 min with 6 μM $AACOCF_3$ reduced the initial rate of a subsequent RVD response, estimated as the water loss during the period 0.8–3.5 min after reduction of the osmolality, from 121 ± 7 fl/min to 89 ± 9 fl/min, i.e., by 24%. Prolonging the preincubation period to 160 min and reducing the cytocrit to 0.5%, to obtain conditions identical to the arachidonic acid release experiments

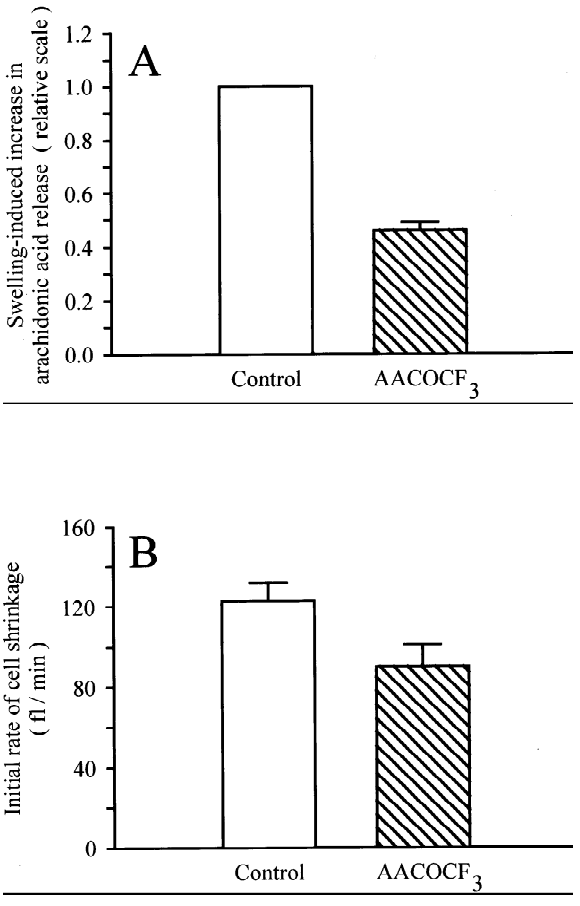

Fig. 5. The effect of $AACOCF_3$ on the swelling-induced ^3H-arachidonic acid release and the RVD response. (*A*) Ehrlich cells were loaded with ^3H-arachidonic acid and the release was followed with time as shown in Fig. 2*A*. During the loading period a fraction of the cell suspension was incubated with 2 μM $AACOCF_3$ for 155–160 min at cytocrit 0.5%. The swelling-induced increase in the presence of $AACOCF_3$ is given relative to the value seen in the absence of the inhibitor. The effect of $AACOCF_3$ was significant ($P < 0.001$). $AACOCF_3$ had no effect on the ^3H-arachidonic acid release under isotonic conditions, i.e., the release was 109 ± 18 cpm per min per 10^6 cells ($n = 6$) in the *absence* of the inhibitor and 111 ± 12 ($n = 6$) in the *presence* of the inhibitor. The cell viability (percentage trypan blue-positive cells) of the cell suspension after 160 min in the presence of 5 μM $AACOCF_3$ was in four experiments estimated at 95 ± 0.2% and 95 ± 0.2% for control cells and cells treated with the inhibitor, respectively. (*B*) Ehrlich cells, cytocrit 1.0%, was preincubated for 45 min with 6 μM $AACOCF_3$. At the end of the pre-incubation period BSA was added (final concentration 0.5%), the cells incubated for another 5 min, whereafter 1.5 ml of the cell suspension was transferred to 100 ml hypotonic medium (157 mOsmol/kg) containing 0.5% BSA. The initial rate of volume recovery, measured as described in Materials and Methods, was 121 ± 7 fl/min ($n = 6$) and 89 ± 9 fl/min ($n = 6$) in control cells and in $AACOCF_3$ treated cells, respectively, i.e., $AACOCF_3$ reduced the rate of water loss significantly to 74% of the control value ($P = 0.036$). Pre-incubation for 90 min or 160 min with $AACOCF_3$ did not improve the inhibition, i.e., the water loss was 75% ($n = 2$) and 61 ± 13% ($n = 3$) of the respective control.

(Fig. 5*A*), and using 5 μM $AACOCF_3$ did not improve the inhibition of the RVD response (39 ± 13%). The RVD responses shown in Fig. 5*B* were performed in the presence of 0.5% BSA, analogous to the release experiments in Fig. 5*A*. Five independent sets of experiments showed that neither the degree of the initial cell swelling following the transfer to hypotonic medium nor the subsequent RVD response were inhibited by the presence of BSA. Actually the 4-min volume recovery was significantly increased from 0.29 ± 0.05 to 0.44 ± 0.02 ($P < 0.03$) by the presence of essentially fatty acid free BSA. Thus, trapping of the arachidonic acid apparently accelerates the RVD response, probably due to an inhibitory effect of arachidonic acid in itself on the volume activated Cl^- channels (Lambert, 1987; Lambert & Hoffmann, 1994).

Possible Role of G-proteins in Activation of PLA_2 and in the RVD Response

Since G-proteins have been reported to be involved in the regulation of the 85 kDa cytosolic phospholipase A_2, either directly or indirectly (*see* Clark et al., 1995), we have investigated the role of G-proteins in the swelling-induced release of arachidonic acid and the RVD response. From Fig. 6*A* and *B* it is seen that the pre-incubation for 30–35 min with 12 μM of the nonhydrolyzable GDP-analogue $GDP_\beta S$ (Eckstein et al., 1979) inhibits the swelling-induced arachidonic acid release by 66% and the RVD response by 19%. This is taken to indicate that a G-protein is involved in the swelling-induced activation of the cytosolic phospholipase A_2 in the Ehrlich cells as well as in the resulting RVD response.

Discussion

Hoffmann and Dunham (1995) presented a model for the signal transduction cascade in Ehrlich cells, activated by cell swelling and leading to activation of channels for K^+, Cl^- and organic osmolytes. According to this model activation of phospholipase A_2 dependent release of arachidonic acid is the initial step in the signal cascade. That phospholipase A_2 activation is a key event in the RVD response was predicted from the observation that inhibitors of phospholipase impaired the RVD response in Ehrlich cells (Lambert & Hoffmann, 1991) as well as in human platelets (Margalit et al., 1993). As emphasized by Strange and coworkers (1996) direct effect of the inhibitors on the channels involved in the RVD response might, however, lead to misinterpretation of studies with inhibitors. It is, therefore important to supply inhibitor studies with direct measurements of the precursors/metabolites involved. The present study was thus initiated to demontrate that arachidonic acid is actually

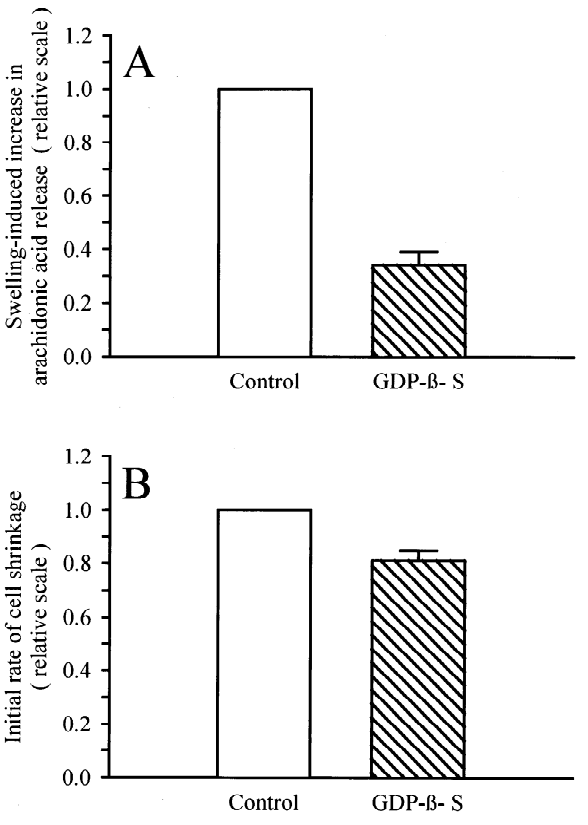

Fig. 6. The effect of $GDP_\beta S$ on the swelling-induced increase in the rate ^{3}H-arachidonic acid release from Ehrlich cells. (*A*) Ehrlich cells were loaded with ^{3}H-arachidonic acid. During the loading period a fraction of the cell suspension was incubated with 12 μM $GDP_\beta S$ for 30–35 min. The cytocrit was 0.5%. Release of ^{3}H-arachidonic acid was followed with time as shown in Fig. 2*A*. The swelling-induced increase in the presence of $GDP_\beta S$ is given relative to the value seen in the absence of the GDP analogue ($n = 5$). In one experiment $GDP_\beta S$ had no effect and this experiment was not included in the calculation of the mean. The effect of $GDP_\beta S$ in the five experiments was significant ($P < 0.001$). (*B*) At the end of the pre-incubation period 2.5 ml of the cell suspension was transferred to 100 ml hypotonic medium (157 mOsmol/kg) and the initial rate of volume recovery was measured and calculated as described in Materials and Methods ($n = 3$). The value is given relative to the value without inhibitor added. The effect of $GDP_\beta S$ was significant ($P = 0.04$).

released during RVD in Ehrlich cells and to characterize the phospholipase(s) activated by the cell swelling.

The data in Figs. 2 and 4 demonstrate that cell swelling is accompanied by a transient increase in the release of ^{3}H-labeled arachidonic acid and in the production of ^{14}C-stearic acid-labeled lysophosphatidylcholine, respectively. An increase in ^{14}C-lyso-PC production could theoretically be due to a decreased lysophospholipase activity or a decreased rates of reacylation/transacylation. However, the concomitant increase in ^{14}C-lyso-PC production and ^{3}H-arachidonic acid-release strongly indicates that a phospholipase A_2 is responsible for the swelling-induced increases. Although we presumably did not label the cells to steady state we assume that the ^{3}H-arachidonic acid and the ^{14}C-stearic acid, which are incorporated in the phospholipids (*see* Table), are attached with some degree of specificity at the *sn*-2 and the *sn*-1 position of the phospholipids, respectively (Allen et al., 1992; Chilton & Murphy, 1987). Arachidonic acid could alternatively also be liberated by a multistep reaction including either (i) phospholipase A_1 followed by a lysophospholipase, (ii) phospholipase C followed by DAG lipase and MAG lipase, or (iii) phospholipase D followed by PAPase, DAG lipase and MAG lipase (Fig. 1). Release of arachidonic acid via these multistep reactions implies in all cases that the fatty acid in the *sn*-1 position, for which we use ^{14}C-stearic acid as a tracer, should be released before release of arachidonic acid can occur (Fig. 1). The data in Fig. 3, which demonstrate that the swelling-induced release of arachidonic acid is not accompanied by a release of stearic acid, argue strongly against the involvement of phospholipase A_1, C and D in the signal cascade activated by cell swelling. Since we see no release of ^{14}C-stearic acid we assume that the lysophospholipase activity of cytosolic phospholipase A_2 is not activated during the initial phase of the RVD response of Ehrlich cells. An additional argument against the involvement of phospholipase C is the lack of effect of the phospholipase C inhibitor U-73122 and the diacylglycerol lipase inhibitor RHC-80267 (*see* Results). Finally, using a well established phospholipase D activity assay, we directly demonstrate that cell swelling did not activate the phospholipase D (*see* Results).

From the discussion above it is concluded that a phospholipase A_2 is activated by cell swelling and responsible for the transient increased liberation of arachidonic acid (Fig. 2). This is in accordance with previous observation that the RVD response in Ehrlich cells is blocked by the phospholipase A_2 inhibitors RO 31-4493 and RO 31-4639 (Lambert & Hoffmann, 1991). According to Henderson, Chappel & Jones (1989) these RO-compounds were designed with special emphasis on the catalytic site of the secreted pancreatic phospholipase A_2, but the specificity towards the different phospholipases has to our knowledge not been investigated. All the secreted types of phospholipase A_2 are dependent of Ca^{2+} in the millimolar range, whereas the cytosolic phospholipases are sensitive to Ca^{2+} in the micromolar range or not sensitive at all (*see* Dennis, 1994). Since the regulatory volume response is unaffected by removal of extracellular Ca^{2+}, we conclude that the phospholipase A_2 activated by cell swelling in the Ehrlich cells is likely to be of the cytosolic type. $AACOCF_3$, which is a cell-permeable trifluoromethyl ketone analogue of arachidonic acid and claimed to be a potent and a selective inhibitor of the 85 kDa, cytosolic phospholipase A_2 (Street et al., 1993), is found to inhibit the swelling-induced increase in arachidonic acid release in Ehrlich

cells (Fig. 5*A*) as well as the subsequent RVD response (Fig. 5*B*). We, therefore, suggest that the 85 kDa cytosolic phospholipase A_2 is activated by cell swelling in Ehrlich cells. It should be noted, that the observation that RO 31-4639 and RO 31-4649 block the RVD response almost completely (Lambert & Hoffmann, 1991), whereas $AACOCF_3$ only blocks the RVD response by about 25–39% (Fig. 5*B*) could well indicate that other types of phospholipase A_2 are also activated by cell swelling. Furthermore, that we do not know whether the RO-compounds could have direct effects on the volume activated channels. From the ability of different phospholipase inhibitors to inhibit the RVD response in human platelets, Margalit and coworkers (1993) demonstrate that a bromophenacyl bromide-, manoalide-sensitive phospholipase A_2 is involved in the signal transduction cascade, activated by cell swelling.

The 85 kDa cytosolic phospholipase A_2 has been demonstrated in several cell types and it seems to participate in several different signaling processes (*see* Clark et al., 1995). Activation of the cytosolic 85 kDa phospholipase A_2 involves MAP kinase phosphorylation (Lin et al., 1993) as well as a Ca^{2+} dependent translocation to the nuclear/endoplasmatic reticulum membranes (Schievella et al., 1995). The Ca^{2+} dependence of the cytosolic phospholipase A_2 is in the submicromolar range (Bauldry et al., 1996; *see* Dennis, 1994; *see* Clark et al., 1995), and the translocation from the cytosol to the membranes might occur in response to small Ca^{2+} signals (*see* Dennis, 1994). We have not been able to demonstrate any increase in the cytosolic Ca^{2+} concentration during the RVD response in the Ehrlich cells (Jørgensen et al., 1997). However, as we state in the paper, the technique we used to estimate free intracellular Ca^{2+} (single-cell measurements on fura-2 loaded cells by fluorescence microscopy with digital image processing) cannot exclude a minor localized increase in $[Ca^{2+}]_i$ close to the endoplasmatic reticulum or the nuclear envelope large enough to activate the 85 kDa cytosolic phospholipase A_2.

It has been suggested that G-proteins are involved in the regulation of the cytosolic type of the phospholipase A_2 (*see* Clark et al., 1995). The nonhydrolyzable GTP-analogue guanosine 5′-O-(3-thiotriphosphate) ($GTP_\gamma S$) stimulates arachidonic acid release in many cells (*see* Clark et al., 1995). In agreement with this, preincubation of the Ehrlich cells with 12 μM $GDP_\beta S$ for 30 to 35 min inhibits the swelling-induced increase in the arachidonic acid release by 66% (Fig. 6). Cells are normally not permeable to $GDP_\beta S$, however, after 30-min incubation with the GDP-analogue a significant inhibition of the RVD response was seen in human platelets (Margalit et al., 1993), which was taken to represent an inhibition of the phospholipase A_2. Therefore, it seems as if $GDP_\beta S$, in at least some cell types, slowly penetrates cell membranes and affect intracellular G-proteins. The inhibition by $GDP_\beta S$ of the swelling-induced release of arachidonic acid and of the RVD response (Fig. 6) is taken to indicate that a G-protein in the Ehrlich cells is involved in the swelling-induced activation of the cytosolic phospholipase A_2 and the concomitant RVD response. Whether the G protein in question interacts directly with the phospholipase or indirectly via a kinase (MAP kinase), as suggested by Clark and coworkers (*see* Clark et al., 1995), is at the present unknown.

From the above we conclude that osmotic cell swelling of the Ehrlich cells activates a phospholipase A_2 — perhaps the cytosolic 85 kDa type — by a partly G-protein coupled process, and that this activation is essential for the subsequent volume regulatory response. Some of the arachidonic acid, released by the phospholipase A_2, may then be oxidized via the 5-lipoxygenase system resulting in the previously measured increase in leukotrienes (Lambert et al., 1987). It should be noted that the prostaglandin synthesis in Ehrlich cells is reduced by the cell swelling simultaneously to the increase in the leukotriene synthesis, i.e., the 5-lipoxygenase pathway is somehow favored after cell swelling at the expense of the cyclooxygenase pathway (Lambert et al., 1987).

Several possibilities for the activation of the cytosolic phospholipase A_2, including (i) changes in the relation between the enzyme, the G protein and the cytoskeleton, (ii) changes in the cytosolic density of proteins (macromolecular crowding), and (iii) opening of nonselective, Ca^{2+} permeable channels (SAC channels), (Christensen & Hoffmann, 1992) leading to localized changes in $[Ca^{2+}]_i$, have been discussed by Hoffmann and Dunham (1995). In addition, Lehtonen and Kinnunen (1995) have demonstrated that changes in the lateral lipid packing by osmotic swelling of large unilamellar liposomes was sufficient to activate snake venom phospholipase A_2, and it was proposed that the membrane bilayer in itself can act as a mechanosensor.

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