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Cell Swelling Activates Separate Taurine and Chloride Channels in Ehrlich Mouse Ascites Tumor Cells

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Abstract. The taurine efflux from Ehrlich ascites tumor cells is stimulated by hypotonic cell swelling. The swelling-activated taurine efflux is unaffected by substitution of gluconate for extracellular Cl^- but inhibited by addition of MK196 (anion channel blocker) and 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS; anion channel and anion exchange blocker) and by depolarization of the cell membrane. This is taken to indicate that taurine does not leave the osmotically swollen Ehrlich cells in exchange for extracellular Cl^-, i.e., via the anion exchanger but via a MK196- and DIDS-sensitive channel that is potential dependent. An additional stimulation of the swelling-activated taurine efflux is seen after addition of arachidonic acid and oleic acid. Cell swelling also activates a "Mini Cl^- channel." The Cl^- efflux via this Cl^- channel, in contrast to the swelling-activated taurine efflux, is unaffected by DIDS and inhibited by arachidonic acid and oleic acid. It is suggested that the swelling-activated "Mini Cl^- channel" and the swelling-activated taurine channel in the Ehrlich cell represent two distinct types of channels.

Key words: Taurine — Anion channel — Indacrinone — Arachidonic acid — Oleic acid — DIDS

Introduction

Taurine (2-ethane sulfonic acid) is a naturally occurring β-amino sulfonic acid (Jacobsen & Smith, 1968), present at high concentrations in Ehrlich ascites tumor cells (53 mM, Hoffmann & Lambert, 1983). The biochemical inertness of taurine, its zwitterionic nature, i.e., poor diffusibility across cell membranes, combined with a high cellular concentration makes taurine well suited as an osmolyte for adjustment of the intracellular osmotic pressure.

The influx of taurine in the Ehrlich cells is at the prevailing taurine concentration in the ascites fluid mediated by a high affinity, Na^+-, Cl^-, and pH-dependent transport system, designated the β-system (Kromphardt, 1963, 1965; Lambert, 1984, 1985; Lambert & Hoffmann, 1993). The β-system is distinctive from other amino acid transporting systems in the Ehrlich cells in that it tolerates substitution of the carboxylate group with a sulfonate or sulfinate group (Lambert, 1985). Taurine influx via the β-system is a 2 Na^+, 1 Cl^-, 1 taurine cotransport, driven by the transmembrane electrochemical Na^+ gradient (Lambert, 1984, 1985). No taurine is taken up in the absence of Na^+ (Lambert, 1984). The 2 Na^+, 1 Cl^-, 1 taurine cotransport is sensitive to the membrane potential due to a negatively charged empty carrier, i.e., depolarization of the cell membrane reduces the taurine influx (*see* Lambert & Hoffmann, 1993). Taurine efflux from Ehrlich cells suspended in isotonic medium is mediated by a Na^+-independent process, stimulated when the cell membrane is depolarized (Lambert, 1984; Lambert & Hoffmann, 1993).

Hypotonically swollen Ehrlich cells recover their cell volume (regulatory volume decrease, RVD) by net loss of K^+ and Cl^- via conductive transport pathways (Hoffmann, Lambert & Simonsen, 1986) and by net loss of taurine via a leak pathway (Hoffmann & Lambert, 1983). The swelling-activated K^+ and Cl^- channels in Ehrlich cells have recently been directly studied by Christensen and Hoffmann (1992), using the patch clamp technique. In the cell-attached mode, a small 3–7 pS Cl^- channel was found to be activated by hypotonic exposure with a time delay of about 1 min (Christensen & Hoff-

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mann, 1992). The open channel density was estimated at $6 \cdot 10^6$ channels per cm^2, and at this density the "Mini Cl^- channel" accounts for the major part of the volume-activated Cl^- efflux (Christensen & Hoffmann, 1992).

According to a model for activation of the RVD response in Ehrlich cells (*see* Hoffmann, Simonsen & Lambert, 1993; Lambert, 1994) it is the membrane deformation or change in the intracellular concentrations of ions or other cytoplasmic components that stimulate a phospholipase A_2 and/or a 5-lipoxygenase and thereby initiate the activation of the K^+, Cl^- and taurine transporting systems. An alternative explanation is that the phospholipase A_2 and the 5-lipoxygenase are activated by Ca^{2+}, which either enters the cell through stretch-activated cation channels or is mobilized from intracellular stores by Ins (1, 4, 5) P_3 (*see* Hoffmann et al., 1993; Lambert, 1994). A mechanical-biochemical transduction mechanism involving a pertussis-sensitive G protein and a phospholipase A_2 has recently been suggested to initiate the RVD response in the human platelets (Margalit et al., 1993). Leukotriene D_4 (LTD_4), a 5-lipoxygenase product of arachidonic acid, seems to act as a second messenger in the Ehrlich cells in the swelling-induced activation of the conductive K^+ and Cl^- transport pathways and of the taurine leak pathway (*see* Lambert, Hoffmann & Christensen, 1987; Lambert, 1989, 1994; Hoffmann et al., 1993; Lambert & Hoffmann, 1993). Thus, the leak pathway has the same activation mechanism as the K^+ and Cl^- channels.

It has previously been demonstrated that the swelling-activated "Mini Cl^- channel" in Ehrlich cells is inhibited by arachidonic acid (20:4) (Lambert, 1987, 1991) and that the effect is mimicked by other unsaturated fatty acids containing *cis* double bonds, i.e., palmitoleic acid (16:1), oleic acid (18:1), linoleic acid (18:2), and linolenic acid (18:3) (Lambert, 1987). The shorthand notation designates the length of the carbon chain and the number of double bonds. Since saturated fatty acids, i.e., stearic acid (18:0), and arachidic acid (20:0) and unsaturated *trans* isomers, i.e., elaidic acid, (18:1) have no effect on the swelling-activated "Mini Cl^- channel" it has been proposed that the inhibitory effect of arachidonic acid on the swelling-activated "Mini Cl^- channel" in Ehrlich cells is caused by a nonspecific detergent effect of an unsaturated fatty acid containing *cis* double bonds (Lambert, 1987).

Recently Strange and coworkers (1993) have demonstrated that arachidonic acid inhibits the swelling-induced inositol and taurine efflux in rat C6 glioma cells and suggested that a putative common transport pathway for taurine and inositol is a volume-sensitive anion channel. Other investigators have reported evidence for taurine movement via anion channels in MDCK cells (Banderali & Roy, 1992; Roy & Malo, 1992), and via Cl^- channels in a human lung cancer cell line (Kirk & Kirk, 1993) and in flounder erythrocytes (Kirk, Ellory & Young, 1992). The question now arises whether the swelling-activated taurine leak in Ehrlich cells is actually mediated via the swelling-activated "Mini Cl^- channel" or whether it is a separate channel, but with an identical activation mechanism.

Materials and Methods

Cell Suspensions and Incubation Media

Ehrlich ascites tumor cells (hyperdiploid strain) were originally identified as the Ehrlich mouse mammary carcinoma (*see* Levinson, 1982). The cell line used in the present work is the ascites type and the culture is maintained by weekly intraperitoneal transplantation in Naval Medical Research Institute (NMRI) mice. Cells for experimental use were harvested eight days after transplantation and suspended in a standard medium containing heparin (2.5 IU/ml). The cells were washed by centrifugation (700 × *g*, 45 sec), once with the standard solution and twice in the appropriate experimental solutions. The temperature was kept at 37°C.

The standard NaCl medium (300 mOsm) had the following composition (mM): 150 Na^+, 5 K^+; 150 Cl^-; 1 Mg^{2+}; 1 Ca^{2+}; 1 sulfate; 1 inorganic phosphate; 3.3 MOPS (3-(*N*-morpholino)propanesulfonic acid); 3.3 TES (*N*-tris-(hydroxy methyl)-methyl-2-aminoethanesulfonic acid; and 5 HEPES (*N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid)). CholineCl medium and KCl medium were made by substitution of choline$^+$ and K^+ for Na^+, respectively, and Nagluconate medium was made by substitution of gluconate for chloride. Hypotonic media (150 mOsm) were prepared by dilution of the isotonic media with distilled water containing the buffers alone. The pH was adjusted to 7.4.

Fluorometric Estimation of the Membrane Potential

Membrane potentials were estimated from the fluorescence intensity of the dye 1,1'-dipropyloxadicarbocyanine ($DiOC_3$-(5)). The dye was added to cell suspensions with a cytocrit of 0.25% at a final concentration of 1.6 μM. Calibration of the fluorescence signal was performed with the cation ionophore gramicidin using cells suspended in Na^+-free, K^+/choline$^+$ media, i.e., standard medium in which NaCl is replaced by KCl and cholineCl and where the sum of K^+ and choline$^+$ is kept constant at 155 mM. The calibration curve was obtained as a plot of the fluorescence *vs.* the Nerst equilibrium potentials for K^+ after addition of gramicidin (*see* Lambert, Hoffmann & Jørgensen, 1989). The fluorometric measurements were performed in polystyrene cuvettes using a Perkin Elmer LS-5 luminescence spectrometer connected to a Perkin Elmer R 100A recorder and a Perkin Elmer CP 100 graphic printer. Excitation and emission wavelengths were 577 and 605 nm, respectively, and slit widths were 5 nm. The temperature of the cuvette was thermostatically controlled (37°C) and the cell suspension was continuously stirred by use of a Teflon-coated magnet, driven by a motor attached to the cuvette house.

Cellular ^{14}C-labeled Taurine and ^{36}Cl Activity

The cellular activity of ^{14}C-labeled taurine and ^{36}Cl was estimated by transferring 1 ml cell suspension (cytocrit 6%) to preweighed vials and separating the cells from the medium by centrifugation (20,000 × *g*, 60 sec). One hundred microliters of the supernatants were diluted 10 times with perchloric acid (7% final concentration) and saved for de-

termination of extracellular taurine activity. Excess supernatant was removed by suction and the wet weight of the cell pellet was determined by reweighing the samples. The packed cells were then lysed in 800 µl distilled water, deproteinized by addition of 100 µl 70% perchloric acid and centrifuged (20,000 × *g*, 10 min). The supernatant was used for determination of cellular $^{14}C/^{36}Cl^-$ activity and the perchloric acid precipitate was dried (90°C, 48 hr) and used for determination of the cell dry weight (*see* Lambert et al., 1989). Cellular $^{14}C/^{36}Cl^-$ activity (cpm/g cell dry weight) was corrected for activity trapped in the extracellular medium using ^{3}H-inulin as marker (Hoffmann, Simonsen & Sjøholm, 1979).

Taurine Efflux

Cells were equilibrated with ^{14}C-labeled taurine (0.5 µCi/ml) for 60 to 100 min. At the end of the preincubation period, duplicate samples were taken for determination of cellular taurine activity (*see above*). For transference of ^{14}C-labeled cells to the efflux medium, 1 ml cell suspension was centrifuged in nylon tubes (ID 3 mm) for 1 min at 770 × *g*, the tube was cut 1 mm below the interface between the packed cells and the medium. The packed cells were then injected into 8 ml of the appropriate medium by flushing the small sleeve of nylon tube containing the packed cells with 1 ml of the medium. The efflux was followed with time by serially isolating cell-free efflux medium by centrifugation of 900 µl cell suspension through a silicone oil phase (300 µl: 1 part 20 AR/1 part AR 200). At the end of the efflux experiment, triplicate samples of the efflux suspension were taken for determination of protein using bovine serum albumin as a standard (Lowry et al., 1951). The amount of protein (g/ml medium) was converted to cell dry weight (g/ml medium) using a protein/dry weight ratio of 0.78 (Hoffmann & Lambert, 1983). The initial activity of ^{14}C-taurine in the cellular pool (a_o^c) was converted from (cpm/g cell dry wt) to (cpm/ml medium) by multiplication with the amount of cell dry weight (g/ml medium).

The rate constant for taurine efflux ($k'c$, $min^{-1} \cdot$ g cell water/g dry wt) was estimated as the product of the cell water content (g/g dry wt) and the slope of a plot of $(a_t^m - a_o^m)/a_o^c$ *vs.* time (*see* Fig. 3*A*), where a_t^m and a_o^m are the ^{14}C-taurine activity in the efflux medium at time t and *zero time*, respectively, and where a_o^c is the cellular ^{14}C-taurine activity at *zero time*. The unidirectional taurine efflux (µmol/g cell dry wt · min) can be calculated from the rate constant by multiplication with the cellular taurine concentration (µmol/g cell water).

$^{36}Cl^-$ Efflux

Cells, equilibrated with $^{36}Cl^-$ (0.45 µCi/ml) in standard medium for 30 to 50 min, were packed by centrifugation and at *zero time* resuspended in the experimental solution. The chloride efflux (µmol/g cell dry wt · min) was calculated from the initial loss in cellular $^{36}Cl^-$ activity (cpm/g cell dry wt · min) divided by the specific activity of cellular chloride (cpm/µmol). The cellular Cl^- concentration was assessed by coulometric titration (CMT 10 chloride titrator, Radiometer, Denmark). Alternatively, the cells were handled and the ^{36}Cl efflux followed as the gain in the medium, as described for the ^{14}C-taurine efflux.

Radioactivity was measured in a liquid scintillation spectrometer (Packard TRI-CARB 460C Liquid Scintillation System) using ULTIMA GOLD™ (Packard) as scintillation fluid.

Cell Volume

Cell volumes were measured on cultures diluted 500 times with filtered media (Millipore, pore size 0.45 µm), resulting in a final cell density of 80,000–100,000 cells/ml (cytocrit ≈ 0.01%). Volumes were calculated as the median of volume distribution curves obtained with a Coulter counter model ZB equipped with a Coulter channelyzer C 1000. Tube orifice was 100 µm. Calibration was carried out with latex beads (diameter 13.5 µm, Coulter Electronics).

Chemicals

Stock solutions of gramicidin (1 mM; Sigma), $DiOC_3$-(5) iodide (1.2 mM; Molecular Probes, Junction City, OR), MK196 (indacrinone, 300 mM, Merck Sharp & Dohme, Herlev, Denmark), arachidonic acid, oleic acid (25 mM, Sigma), and bumetanide (5 mM, Sigma) were prepared in ethanol. Stock solutions of DIDS (20 mM, Sigma) were prepared in standard medium. BSA (bovine serum albumin, Sigma) was used as protein standard. Silicone oils AR20 and AR200 were from Wacker Chemie (Vienna, Austria). ^{3}H-inulin, ^{14}C-taurine and ^{36}Cl were obtained from New England Nuclear.

Statistical Evaluation

All values are given as mean values ± SE of the mean unless otherwise stated. Paired Student's *t*-test was used to evaluate statistical significance.

Results

Effect of Anion Transport Inhibitors on Regulatory Volume Decrease in the Presence of Gramicidin in Na^+-free Media

When the K^+ channel is bypassed by addition of gramicidin in Na^+-free media, the rate of cell shrinkage can be used to evaluate the effect of inhibitors on the swelling-activated Cl^- permeability (*see* Hoffmann et al., 1986). Figure 1 shows that the RVD response in Ehrlich cells in hypotonic Na^+-free medium ($choline^+$ substituted for Na^+) is inhibited by MK196 (indacrinone, Fig. 1*A*), a Cl^- channel blocker (Distefano et al., 1985; Durr & Larsen, 1986) and by arachidonic acid (Fig. 1*B*). The concentration required for 50% inhibition of the swelling-activated Cl^- channel (IC_{50}) is estimated at about 0.5 mM and 5 µM for MK196 and arachidonic acid, respectively. On the other hand, DIDS, which is known to inhibit anion exchange and some anion channels (*see* Cabantchik & Greger, 1992; Gunn, 1992), has only a marginal effect on the swelling-induced Cl^- permeability (Fig. 1*C*). Although the rate of the RVD response under the present experimental condition is dominated by the Cl^- permeability, we have previously shown that organic osmolytes, in particular taurine, contribute to the volume regulation (Hoffmann & Lambert, 1983). The following experiments were therefore designed to characterize the swelling-activated Cl^- transport pathway as well as the swelling-activated taurine transport pathway.

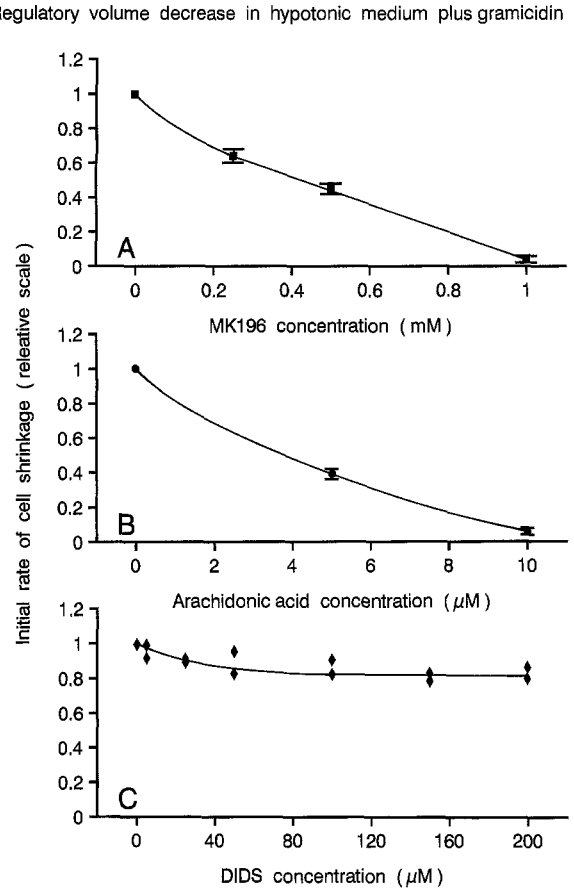

Fig. 1. Effect of MK196, arachidonic acid and DIDS on the swelling-activated Cl^- channel. Cells were swollen in hypotonic, Na^+-free cholineCl medium and the cell volume was followed with time in a Coulter counter. The final cytocrit was about 0.01%. MK196 (0.25 to 1 mM, *A*), arachidonic acid (5–10 μM, *B*) or DIDS (5 –200 μM, *C*) were added at the time of hypotonic exposure. Gramicidin (0.5 μM) was added at the time of maximal cell swelling (at $t = 0.9$) to ensure a high K^+ permeability. The *initial rate of cell shrinkage* (fl/min) was calculated as the water loss (fl) within 1.5 min after addition of gramicidin. The rate of cell shrinkage is given relative to the rate measured in hypotonic control cells with gramicidin but without any drug added. Values in *A* and *B* are means of three sets of experiments. The data in *C* represent two sets of experiments. The initial rate of volume recovery in the presence of 200 μM DIDS was estimated at 0.89 ± 0.06 in four experiments (relative values).

Inhibitors of the Swelling-Activated Cl^- Efflux

Figure 2 demonstrates that the $^{36}Cl^-$ efflux in Ehrlich cells in hypotonic Cl^--free medium is almost completely inhibited by MK196 (2 mM) and by arachidonic acid (200 μM). The experiments were performed in Cl^--free medium (gluconate substituted for chloride) and in the presence of bumetanide to block $^{36}Cl^-$ efflux via the anion exchanger and the Cl^--dependent cotransport systems, respectively. The higher MK196 and arachidonic acid concentration used for the assessment of $^{36}Cl^-$ efflux was chosen to compensate for an increased drug partition into the membrane due to the higher cytocrit (3% in $^{36}Cl^-$ efflux experiments compared to 0.01% in cell volume measurements).

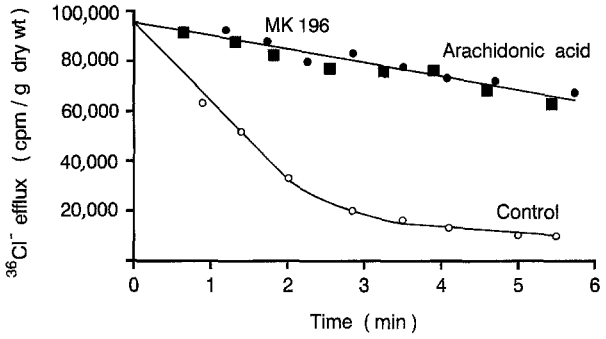

Fig. 2. Effect of MK196 and arachidonic acid on the swelling-activated Cl^- efflux. Ehrlich cells, equilibrated with $^{36}Cl^-$ in standard NaCl medium, were transferred at time zero to hypotonic, Cl^--free gluconate medium containing 30 μM bumetanide, and the $^{36}Cl^-$ efflux was followed with time. The final cytocrit was about 3%. MK196 (2 mM, filled squares) and arachidonic acid (200 μM, filled circles) were added at the time of hypotonic exposure. *$^{36}Cl^-$ efflux* is shown as the loss in cellular $^{36}Cl^-$ activity (cpm/g cell dry wt). The curves are representative of three sets of experiments.

Swelling-Activated Taurine Efflux

The swelling-activated taurine efflux is not an exchange of cellular taurine for extracellular Cl^- via the anion exchange system. This is deduced from Fig. 3*A* where it is shown that the taurine efflux is similar in hypotonic Cl^--containing and in Cl^--free medium (gluconate substituted for chloride). Figure 3*B* shows that the rate constant for taurine efflux is increased fourfold after swelling in hypotonic media with half osmolarity, and that the rate constant in hypotonic Cl^--free medium is not significantly different from the rate constant in hypotonic Cl^--containing medium ($P < 0.05$).

Figure 4 demonstrates the effect of membrane depolarization on the swelling-activated taurine efflux as well as the cell volume 0.8 min after hypotonic exposure. The cells were transferred to hypotonic, Na^+-free media with varying extracellular K^+ concentration, and gramicidin was added at the time of hypotonic exposure to clamp the membrane potential at the Nernst equilibrium potential for K^+ (*see* Lambert et al., 1989). From Fig. 4 it is seen that depolarization of the cell membrane reduces the rate constant for taurine efflux in hypotonically swollen Ehrlich cells. This is in contrast to the rate constant for taurine efflux in Ehrlich cells suspended in isotonic medium, which is increased by membrane depolarization (Lambert & Hoffmann, 1993). This confirms our previous notion (Lambert & Hoffmann, 1993) that the taurine

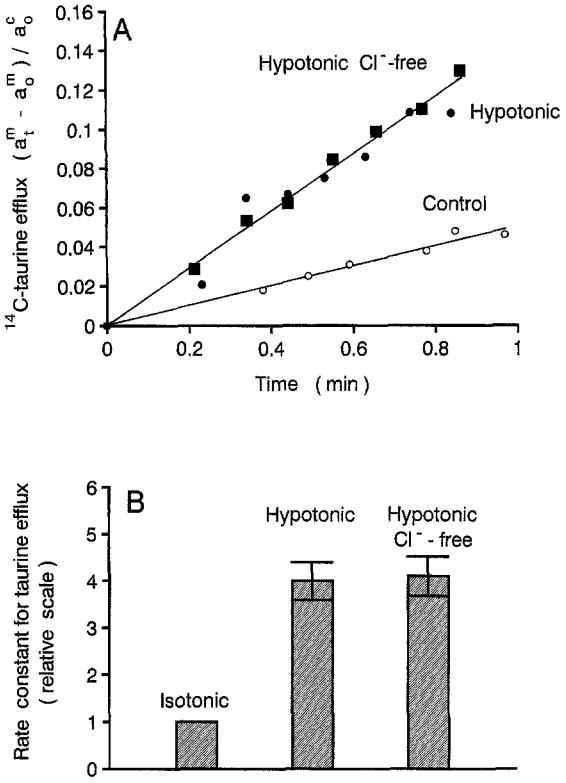

Fig. 3. Effect of substitution of gluconate for extracellular chloride on the rate constant for the swelling-activated taurine efflux. Ehrlich cells, equilibrated with ^{14}C-taurine in standard NaCl medium, were transferred at time zero to isotonic NaCl medium (*A*, open circles), hypotonic NaCl medium (*A*, filled circles) or hypotonic, Cl^--free gluconate medium (*A*, filled squares), and the release of ^{14}C-taurine was followed with time. The final cytocrit was about 0.7%. *Taurine efflux* is shown as the relative specific activity $(a_t^m - a_o^m)/a_o^c$ plotted *vs.* the time, where a_t^m and a_o^m are the ^{14}C-taurine activity in the efflux medium at time t and *zero time*, respectively, and where a_o^c is the cellular ^{14}C-taurine activity at *zero time*. *Rate constants* for taurine efflux (*k'c, B*), calculated as the product of the slope of taurine efflux curves (*A*) and the cell water content, are given relative to the rate constant in isotonic NaCl medium (0.19 ± 0.02 min^{-1} · g cell water/g dry wt). Data represent four sets of experiments.

efflux system, which dominates after cell swelling, is different from the taurine efflux system, which dominates under isotonic conditions. It is noted that the cellular permeability to taurine in Ehrlich cells increases as a function of increasing cell volume (Hoffmann & Lambert, 1983). However, the cell volume in the presence of gramicidin increases with increasing concentrations of extracellular K^+, whereas the taurine efflux decreases (*see* Fig. 4). The effect of membrane depolarization seen in Fig. 4 might therefore be slightly underestimated. The inhibition of the taurine efflux after depolarization is in agreement with the idea that taurine during RVD either leaves the Ehrlich cells as an anion or via a channel, which is potential dependent, i.e., inhibited by depolarization of the cell membrane.

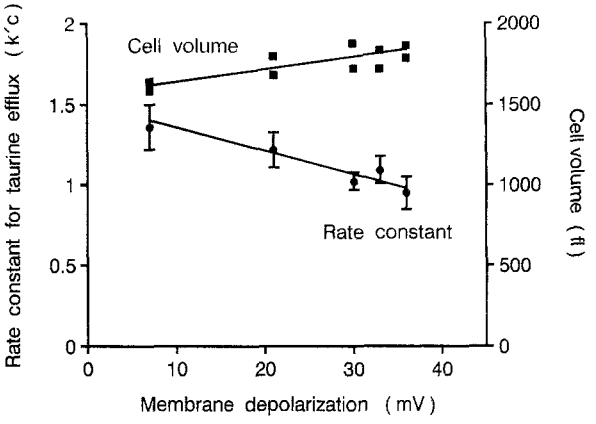

Fig. 4. Effect of the cell membrane potential on the rate constant for the swelling-activated taurine efflux. Ehrlich cells, equilibrated with ^{14}C-taurine for 60 min in standard NaCl medium, were transferred at time zero to hypotonic, Na^+-free K^+/choline$^+$ media in which the extracellular K^+ concentration was varied between 5 to 55 mM and the sum of K^+ plus choline$^+$ kept constant at 75 mM. Gramicidin (10 μM) was added at the time of hypotonic exposure to clamp the membrane potential at the K^+ equilibrium potential. *Rate constants* for taurine efflux (*k'c*, min^{-1} · g cell water/g dry wt, filled circles), estimated as described in the legend to Fig. 3, are given as the mean of three sets of experiments. *Cell volumes* (fl, filled squares), measured 0.8 min after transfer to the hypotonic medium, represent two sets of experiments. *Membrane potentials* (mV) were estimated from the fluorescence of the potential sensitive probe $DiOC_3$ – (5) in separate experiments.

Comparison of the Swelling-activated Cl^- Efflux and the Swelling-activated Taurine Efflux

Figure 5 compares the effect of different anion transport inhibitors on the Cl^- efflux and the taurine efflux in Ehrlich cells after cell swelling in hypotonic, Cl^--free medium (gluconate substituted for chloride). It is seen that the anion channel blocker MK196 inhibits the Cl^- efflux by $84 \pm 5\%$ (Fig. 5*A*) and the taurine efflux by $67 \pm 3\%$ (Fig. 5*B*). DIDS, which inhibits anion exchange and some anion channels (*see above*), is found to inhibit the Cl^- efflux only by $29 \pm 6\%$ (Fig. 5*A*), whereas the taurine efflux is inhibited by $71 \pm 4\%$ (Fig. 5*B*). The taurine efflux is in the presence of both MK196 and DIDS reduced to the level found in Ehrlich cells suspended in isotonic medium. Arachidonic acid (200 μM) inhibits the Cl^- efflux in osmotically swollen cells by $68 \pm 3\%$ (Fig. 5*A*), whereas the fatty acid (25 μM) stimulates the swelling-activated taurine efflux by $72 \pm 15\%$ (Fig. 5*B*).

To ensure that the difference in the effect of arachidonic acid on Cl^- efflux and taurine efflux was not caused by differences in experimental conditions and procedures, we measured the Cl^- efflux in parallel to the taurine efflux, using the same batch of cells, the same cytocrit and exactly the same procedure as for the taurine

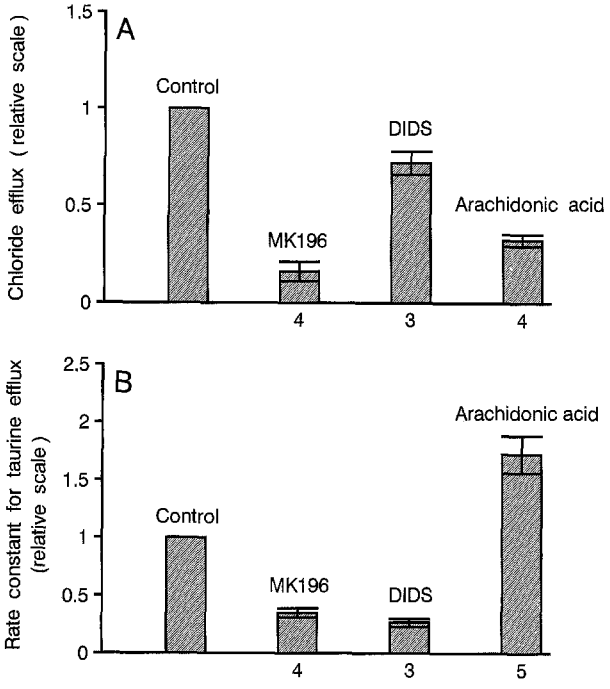

Fig. 5. Effect of MK196, DIDS and arachidonic acid on the swelling-activated Cl^- efflux and the rate constant for the swelling-activated taurine efflux. $^{36}Cl^-$ efflux (*A*) was measured in hypotonic, Cl^--free gluconate medium as described in Fig. 2 (cytocrit about 3%). DIDS (300 μM), MK196 (2 mM) or arachidonic acid (200 μM) were added at the time of hypotonic exposure. *Chloride efflux,* calculated as the initial loss of cellular $^{36}Cl^-$ activity (cpm/g cell dry wt per min) divided by the specific activity for Cl^- in the cell (cpm/μmol), is given relative to the efflux measured in hypotonic medium with no drugs added (49 ± 2 μmol/g dry wt · min, n = 5). ^{14}C-taurine efflux (*B*) was measured in hypotonic, Cl^--free gluconate medium (cytocrit about 0.7%). DIDS (200 μM), MK196 (1 mM) or arachidonic acid (25 μM) were added at the time of hypotonic exposure. *Rate constants* for taurine efflux, estimated as described in the legend to Fig. 3, are given relative to the rate constant measured in hypotonic gluconate medium with no drugs added (0.73 ± 0.06 min^{-1} · g cell water/g dry wt, n = 8). The number of paired experiments is indicated below the bars.

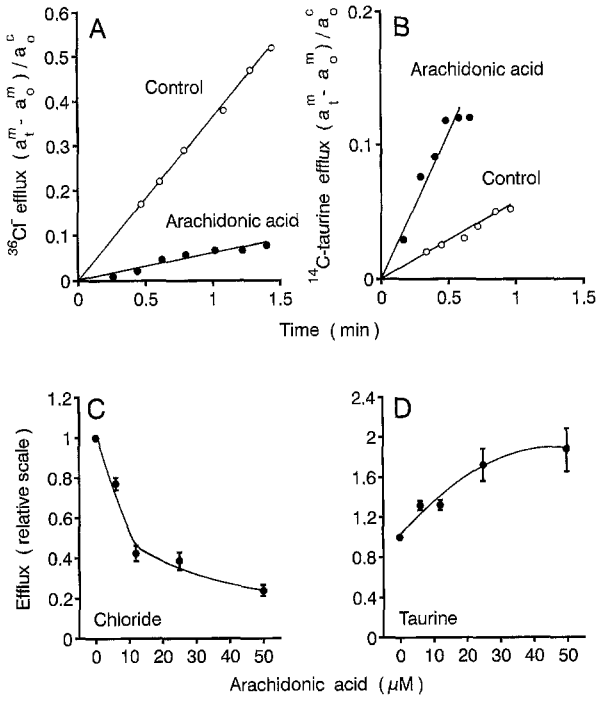

Fig. 6. Effect of arachidonic acid on the swelling-activated Cl^- channel and the swelling-activated taurine channel. Cells were loaded with $^{36}Cl^-$ or ^{14}C-taurine and the efflux was measured in hypotonic, Cl^--free gluconate medium containing 30 μM bumetanide using the technique described for the taurine efflux. The final cytocrit was in both cases 0.7%. Arachidonic acid (0 to 50 μM) was added at the time of hypotonic exposure. *$^{36}Cl^-$ efflux* (*A*) and the *^{14}C-taurine efflux* (*B*) are shown as the relative specific activity, $(a_t^m - a_o^m)/a_o^c$ plotted *vs.* the time, where a_t^m and a_o^m are the activity in the efflux medium at time t and *zero time,* respectively, and where a_o^c is the cellular activity at *zero time. Rate constants* for $^{36}Cl^-$ efflux (*C*) and ^{14}C-taurine efflux (*D*) in arachidonic acid treated cells were estimated as described in Fig. 3 and given relative to the rate constant measured in hypotonic medium in the absence of arachidonic acid. Values in *A* and *B* represent five sets of experiments, whereas values in *C* and *D* represent the mean of three sets of experiments.

efflux. Figure 6 confirms that arachidonic acid (25 μM) has opposite effects on the swelling-activated Cl^- efflux (Fig. 6*A*) and the swelling-activated taurine efflux (Fig. 6*B*). Figure 6 also presents the concentration dependence curves for the effect of arachidonic acid on the initial rate of Cl^- efflux (Fig. 6*C*) and the initial rate of taurine efflux (Fig. 6*D*). It is seen that arachidonic acid inhibits the Cl^- efflux and stimulates the taurine efflux in the whole range from 5 to 50 μM arachidonic acid. In two sets of experiments it was demonstrated that oleic acid (25 μM) reduced the swelling-activated Cl^- efflux by 70% (n = 2), whereas oleic acid (40 μM) stimulated the swelling-activated taurine efflux by 56% (n = 2). Since oleic acid is not metabolized into arachidonic acid or any eicosanoids in mammalian cells, it is proposed that the effect of arachidonic acid on the swelling-activated chloride and taurine transporting systems represents an effect of an unsaturated fatty acid.

The effect of MK196 and DIDS on the swelling-activated taurine efflux (Fig. 5*B*), together with the dependence of the taurine efflux on the membrane potential (Fig. 4), is taken to indicate that taurine in hypotonically swollen Ehrlich cells leaves via a MK196- and DIDS-sensitive channel which is potential sensitive. The opposing effects of arachidonic acid on Cl^- and taurine transport and the weak effect of DIDS on the Cl^- efflux after hypotonic cell swelling (Figs. 5 and 6) demonstrate that the dominating swelling-activated Cl^- channel, the "Mini Cl^- channel," is different from the swelling-activated taurine channel.

Discussion

Taurine Efflux after Osmotic Cell Swelling

Hypotonically swollen Ehrlich cells tend to reduce their cell volume towards the initial value by net loss of os-

molytes and cell water (regulatory volume decrease, RVD). About 30% of the total loss of cellular osmolytes during RVD is accounted for by amino acids, predominantly taurine (Hoffmann & Hendil, 1976). The taurine efflux from Ehrlich cells is increased fourfold by osmotic cell swelling in hypotonic media with half osmolarity (Fig. 3), and the cellular taurine concentration is reduced from 53 to 7 mM within 40 min after transfer to the hypotonic medium (Hoffmann & Lambert, 1983). The initial taurine efflux after cell swelling in hypotonic medium with half osmolarity has been estimated at 11 μmol/g cell dry wt · min (Hoffmann & Lambert, 1983), whereas the initial net Cl^- efflux has been estimated at 49 μmol/g cell dry wt · min (*see* legend to Fig. 5). The initial net K^+ efflux has been previously demonstrated to exceed the net Cl^- loss by a factor 1.6 (Hendil & Hoffmann, 1974) and can accordingly be estimated at 78 μmol/g cell dry wt · min. Thus, for the initial RVD followed in Fig. 1, taurine accounts for less than 10% of the osmolyte loss.

The swelling-activated taurine efflux is reduced by depolarization of the cell membrane (Fig. 4), whereas the taurine efflux system in Ehrlich cells suspended in isotonic medium is increased by depolarization of the membrane (Lambert & Hoffmann, 1993). Furthermore, the pH dependence of taurine efflux from Ehrlich cells under hypotonic conditions is different from the pH dependence of taurine efflux under isotonic conditions (*see* Lambert & Hofmann, 1993). This supports our previous notion that the swelling-activated taurine efflux pathway is different from the taurine transporting systems working under isotonic conditions (Hoffmann & Lambert, 1983; Lambert, 1985). An osmolarity-sensitive taurine efflux pathway different from the high affinity β-system has also been demonstrated in rat astrocytes (Kimelberg et al., 1990), in MDCK-cells (Sánchez-Olea et al., 1991), in cerebellar granule cells (Schousboe et al., 1991), and in flounder erythrocytes (Thoroed & Fugelli, 1994).

The Swelling-activated Taurine Efflux is Likely to Be Via an Anion Channel

A common characteristic of the swelling-activated taurine efflux is the sensitivity to DIDS and various anion transport blockers (*see e.g.,* Pasantes-Morales & Schousboe, 1989; Goldstein, Brill & Freund, 1990; Kimelberg et al., 1990; Pasantes-Morales, Moran & Schousboe, 1990; Sánchez-Olea et al., 1991; Schousboe et al., 1991; Ballatori & Boyer, 1992; Kirk et al., 1992; Roy & Malo, 1992; Thoroed & Fugelli, 1994). The swelling-activated taurine efflux in Ehrlich cells is also inhibited by DIDS and MK196 (Fig. 5), but unaffected by substitution of gluconate for extracellular Cl^- (Fig. 3). This is taken to indicate that taurine does not leave the osmotically swollen Ehrlich cells in exchange for extracellular Cl^-, i.e., via the anion exchange system (band 3), as suggested for taurine efflux from skate erythrocytes (Goldstein & Brill, 1991), but rather via a DIDS-sensitive anion channel.

Depolarization of the cell membrane decreases the rate constant for taurine efflux in hypotonically swollen Ehrlich cells (Fig. 4). This is taken to indicate that taurine during RVD either leaves the Ehrlich cells as an anion or via a channel which is potential dependent, i.e., inhibited by depolarization of the cell membrane. If taurine behaves as an anion, a large acidification of the cells would be expected in order to account for the great net loss of taurine during RVD (Hoffmann & Lambert, 1993). However, Livne and Hoffmann (1990) have demonstrated that only a minor acidification takes place during RVD and that the acidification is a result of a redistribution of Cl^- and HCO_3^- via the Cl^-/HCO_3^- exchanger. It is therefore likely that taurine leaves the osmotically swollen Ehrlich cell as a neutral molecule. Our working hypothesis is that the taurine channel in Ehrlich cells is an unselective, potential-dependent anion channel, permeable to some neutral substances as well as to anionic molecules. An anion channel has also been suggested to be involved in the swelling-activated loss of taurine in MDCK cells (Banderali & Roy, 1992; Roy & Malo, 1992), flounder erythrocytes (Kirk et al., 1992) and C6 glioma cells (Strange et al., 1993). The swelling-activated anion channel in MDCK cells has a relatively high permeability to taurine, glutamate, and aspartate and appears to be outwardly rectifying (Banderali & Roy, 1992).

It has been suggested that the swelling-induced activation of the taurine channel and the "Mini Cl^- channel" in Ehrlich cells involves release of arachidonic acid from the membrane phospholipids and an increased oxidation of arachidonic acid into leukotrienes via the 5-lipoxygenase pathway (*see* Hoffmann et al., 1993; Lambert, 1994). The 5-lipoxygenase product LTD_4 seems to act as a second messenger for the activation of the taurine channel as well as for the activation of the "Mini Cl^- channel" (*see* Lambert et al., 1987; Lambert, 1989, 1994; Hoffmann et al., 1993; Lambert & Hoffmann, 1993). LTD_4 is known to mobilize Ca^{2+} (Lambert, 1994) and to depolarize the cell membrane in Ehrlich cells (Lambert, 1989). However, the effect of LTD_4 on taurine efflux in osmotically swollen Ehrlich cells (Lambert & Hoffman, 1993) does not seem to involve a LTD_4-induced increase in cellular Ca^{2+} or a LTD_4-induced depolarization of the cell membrane, because taurine efflux in osmotically swollen Ehrlich cells is higher in Ca^{2+}-free than in Ca^{2+}-containing media (Lambert & Hoffmann, 1993) and because depolarization of the plasma membrane actually reduces the taurine efflux (Fig. 4).

From the present data it is seen that arachidonic acid stimulates taurine efflux in osmotically swollen Ehrlich cells and that the effect is mimicked by oleic acid (*see* Figs. 5*B*, 6*B* and *D*, and Results). Oleic acid is not me-

Table. Characteristics of the "Mini Cl^- channel" and the taurine channel in Ehrlich cells

	"Mini Cl^- channel"	Taurine channel
Conductance		
Cl^- conductance (net Cl^- efflux)[a]	41 ± 5 μS/cm^2	$\leq 2 \pm 1$ μS/cm^2
Single channel conductance[b]	3–7 pS	Unknown
Delay of activation[b]	100 ± 60 sec	<20 sec
Activation/Inhibition		
Cell swelling	Activation[b]	Activation[c]
Membrane depolarization	**No effect**[b]	**Inhibition**[c]
RO 31-4639[d] (phospholipase A_2 inhibitor)	Inhibition	Inhibition
Pimozide[d] (calmodulin blocker)	Inhibition	Inhibition
NDGA and ETH 615-139[d] (5-lipoxygenase inhibitors)	Inhibition	Inhibition
LTD_4[d]	Activation	Activation
PGE_2[d]	No effect	No effect
MK196[c]	Inhibition	Inhibition
DIDS[c]	**Weak inhibition**	**Inhibition**
Arachidonic acid, Oleic acid[c]	**Inhibition**	**Activation**

[a] The Cl^- conductance was calculated from the diffusional Cl^- efflux, the membrane potential and the Nernst equilibrium potential. The Cl^- conductance through the "Taurine channel" is the Cl^- conductance in hypotonic medium (150 mOsm) in the presence of arachidonic acid. The Cl^- conductance through the "Mini Cl^- channel" is the Cl^- conductance in hypotonic medium (150 mOsm) in the absence of arachidonic acid minus the contribution from the taurine channel (Lambert, 1991).
[b] Data for the "Mini Cl^- channel" from Christensen and Hoffman (1992).
[c] Data from the present paper.
[d] Results from Lambert and Hoffmann (1993); Lambert (1994).

tabolized into eicosanoids in mammalian cells, indicating that the stimulating effect of arachidonic acid on taurine efflux represents an effect of an unsaturated fatty acid, although an effect mediated by an increased synthesis of eicosanoids e.g., LTD_4 cannot be excluded. Accordingly, arachidonic acid is regarded as a second messenger for the swelling-induced activation of the taurine channel in Ehrlich cells. Fatty acid-induced activation of taurine channels may have relevance in pathological states, notably in ischemia, which results in cytotoxic cell swelling and where circulating levels of fatty acids are elevated (Bazan, 1970; Katz & Messineo, 1981).

From the similarities between the activation mechanisms for the swelling-activated "Mini Cl^- channel" and the swelling-activated taurine channel (*see* Table), the question arose whether the taurine channel was actually the "Mini Cl^- channel" or whether it was a separate channel, but with an identical activation mechanism.

The Swelling-activated "Mini Cl^- Channel"

A chloride channel with low spontaneous activity has been observed in excised inside-out patches from Ehrlich cells (Christensen & Hoffmann, 1992). The channel activity is not dependent on internal Ca^{2+}, and is not activated by membrane stretch (suction). Single channel currents with characteristics similar to those seen in the isolated patches were also observed in cell-attached patches under isotonic conditions. The single channel conductance of the fully activated channel, rarely seen under isotonic conditions, has been estimated at 3–7 pS (the "Mini Cl^- channel," *see* Table). Channels with a 3 pS conductance have been proposed to represent a partly activated state of the 7 pS channel (Christensen & Hoffmann, 1992). However, in the cell-attached mode the channel is in most patches activated by hypotonic exposure, with a single channel conductance at 7 pS and a time delay of about 1 min (*see* Table). The "Mini Cl^- channel" in Ehrlich cells appears to be an unspecific anion channel, permeable to Cl^-, Br^-, NO_3^-, and SCN^- (Hoffmann et al., 1986).

Other Cl^- Channels

Two other Cl^- channels have been demonstrated using the patch clamp technique in Ehrlich cells — a 400 pS ("Maxi Cl^- channel") and a 34 pS chloride channel ("Medium Cl^- channel") (Christensen & Hoffmann, 1992). The "Maxi Cl^- channel" is voltage dependent, i.e., activated by depolarization of the cell membrane, but it is not directly activated by Ca^{2+} (Christensen & Hoffmann, 1992). The "Medium Cl^- channel" is an inwardly rectifier and appears to have properties similar to the Cl^- channel described in the apical membrane in human airway epithelial cells (Welsh, 1986), which is

activated by cAMP-dependent protein kinase C (Li et al., 1989). Neither the "Maxi Cl^- channel" nor the "Medium Cl^- channel," however, seem to play a role in the RVD response in Ehrlich cells (Christensen & Hoffmann, 1992).

Comparison of the Swelling-activated Cl^- Efflux and the Swelling-activated Taurine Efflux

The Table is a summary of the different characteristics for the "Mini Cl^- channel" and the taurine channel activated in Ehrlich cells by cell swelling in hypotonic medium. There is an identity in the activation mechanisms for the dominating, swelling-activated "Mini Cl^- channel" and the swelling-activated taurine channel, i.e., the sensitivity towards phospholipase A_2 inhibition, 5-lipoxygenase inhibition, the anticalmodulin drug pimozide, LTD_4, and PGE_2. The major differences are (i) the opposite effects of unsaturated fatty acids (arachidonic and oleic acid); (ii) the strong inhibition of the taurine channel produced by DIDS, whereas DIDS has only a weak effect on the Cl^- channel; (iii) the inhibition of the taurine channel following depolarization of the cell membrane, whereas the Cl^- channel is unaffected by the cell membrane potential.

Arachidonic acid has been previously shown to activate an unspecific anion transport pathway in Ehrlich cells suspended in isotonic medium, which accepts Cl^-, as well as propionate (Lambert, 1991). Arachidonic acid also stimulates taurine efflux in Ehrlich cells suspended in isotonic medium (*data not shown*). It is therefore suggested that the channel activated by arachidonic acid accepts inorganic anions, as well as small organic anions, e.g., taurine. The total Cl^- conductance after cell swelling would then represent fluxes via the "Mini Cl^- channel" and via the taurine channel. The total Cl^- conductance after swelling in hypotonic medium with half osmolarity is in the absence and presence of arachidonic acid, estimated at 43 ± 5 $\mu S/cm^2$ and 2 ± 1 $\mu S/cm^2$, respectively (Table 1; Lambert, 1991). The contribution to the total Cl^- conductance from the taurine channels is accordingly 2 $\mu S/cm^2$, or less if the "Mini Cl^- channels" are only partially blocked, whereas the contribution from the "Mini Cl^- channel" is 41 $\mu S/cm^2$ (Table). Thus, unless the taurine channels are present with only a few copies per cell, its Cl^- conductance must be quite low. This excludes the "Medium Cl^- channel" and the "Maxi Cl^- channel" as candidates. An additional Cl^- channel, however, is occasionally seen in the cell-attached mode during RVD in Ehrlich ascites tumor cells (Finn Jørgensen, Lene Jakobsen and Else Hoffmann, *unpublished*). The sensitivity of this channel towards arachidonic acid and DIDS is now under investigation.

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