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Ca^{2+}-mediated Potentiation of the Swelling-induced Taurine Efflux from HeLa Cells: On the Role of Calmodulin and Novel Protein Kinase C Isoforms

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Abstract. The present work sets out to investigate how Ca^{2+} regulates the volume-sensitive taurine-release pathway in HeLa cells. Addition of Ca^{2+}-mobilizing agonists at the time of exposure to hypotonic NaCl medium augments the swelling-induced taurine release and subsequently accelerates the inactivation of the release pathway. The accelerated inactivation is not observed in hypotonic Ca^{2+}-free or high-K^+ media. Addition of Ca^{2+}-mobilizing agonists also accelerates the regulatory volume decrease, which probably reflects activation of Ca^{2+}-activated K^+ channels. The taurine release from control cells and cells exposed to Ca^{2+} agonists is equally affected by changes in cell volume, application of DIDS and arachidonic acid, indicating that the volume-sensitive taurine leak pathway mediates the Ca^{2+}-augmented taurine release. Exposure to Ca^{2+}-mobilizing agonists prior to a hypotonic challenge also augments a subsequent swelling-induced taurine release even though the intracellular Ca^{2+}-concentration has returned to the unstimulated level. The Ca^{2+}-induced augmentation of the swelling-induced taurine release is abolished by inhibition of calmodulin, but unaffected by inhibition of calmodulin-dependent kinase II, myosin light chain kinase and calcineurin. The effect of Ca^{2+}-mobilizing agonists is mimicked by protein kinase C (PKC) activation and abolished in the presence of the PKC inhibitor Gö6850 and following downregulation of phorbol ester-sensitive PKC isoforms. It is suggested that Ca^{2+} regulates the volume-sensitive taurine-release pathway through activation of calmodulin and PKC isoforms belonging to the novel subclass (nPKC).

Key words: Histamine — Bradykinin — H_1-receptor — PMA — Regulatory Volume Decrease

Introduction

Regulation of cell volume is essential for cell survival, as changes in cell volume and activity of volume-regulating osmolyte channels are integrated parts of the regulation of cell physiological functions, e.g., cell cycle progression, gene expression, metabolism, hormonal secretion, membrane transport, and apoptosis (Lang et al., 1998a; Lang et al., 2000b; Eggermont et al., 2001). Consequently, most mammalian cells possess the capacity to adjust their intracellular content of osmolytes and cell volume upon exposure to changes in the intracellular or extracellular osmolarity (Hoffmann & Dunham, 1995; Lang, Busch & Volkl, 1998b).

Cells exposed to a reduction in the extracellular osmolarity swell due to the osmotic equilibrium of water across the plasma membrane and subsequently activate volume-sensitive pathways permeable for K^+, Cl^- and organic osmolytes. This results in a cellular loss of KCl and organic osmolytes concomitant with an osmotically obliged loss of cell water leading to a reduction of the cell volume, a process referred to as regulatory volume decrease, RVD (Lang et al., 1998a). Taurine—2-aminoethane sulfonic acid—is a metabolically inert β-amino acid and an important organic osmolyte in most mammalian tissues, i.e., release of taurine is often associated with a reduction in cell volume following hypotonic cell swelling (Lambert, 2004).

In most cell types, including HeLa cells, the swelling-induced loss of intracellular KCl is mediated

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by separate K^+ and Cl^- channels (Lang et al., 1998b), whereas the leak pathway mediating the loss of organic osmolytes, such as taurine, is in dispute, primarily due to the uncertainty of the molecular identity of the swelling-activated pathways. The swelling-induced pathway for the loss of taurine and the electrophysiologically described volume-activated Cl^--channel, $I_{Cl,vol}$, have several characteristics in common, e.g., non-hydrolytic dependence of intracellular ATP and inhibition by pharmaceutical anion channel blockers. As it can be demonstrated that $I_{Cl,vol}$ is readily permeable for taurine, it has been assumed that the swelling-induced loss of taurine and Cl^- is mediated by one common pathway, which is consequently denoted VSOAC (volume-sensitive organic and anion channel) (Strange, Emma & Jackson, 1996; Nilius et al., 1997; Okada, 1997). However, studies on, e.g., Ehrlich ascites tumor cells, neuroblastoma CHP-100 cells, erythrocytes, mammary tissue, placenta, NIH3T3 fibroblast cells, HeLa cells and glial cells indicate that differences exist for the swelling-induced release of anions and organic osmolytes with respect to pharmacological inhibitor profile and time of activation/inactivation (Lambert & Hoffmann, 1994; DavisAmaral, Musch & Goldstein, 1996; Moran et al., 1997a; Stutzin et al., 1997, 1999; Basavappa et al., 1998; Shennan, 1999; Bres et al., 2000; Shennan & Thomson, 2000). Furthermore, diversity in the signal transduction events leading to the release of Cl^- and taurine, respectively, have been demonstrated in various cell types, i.e., Junankar and co-workers (Junankar, Karjalainen & Kirk, 2002) found that the hypotonic-induced taurine release from HTC liver cells is submitted to regulation by P2 Y-receptors, whereas the I^- efflux (I^- is used as surrogate for Cl^-) is unaffected by modulations in cellular calcium. In Jurkat lymphocytes, the RVD response and activity of $I_{Cl,vol}$ are dependent on the presence of the tyrosine kinase $p56^{lck}$ and is thus inhibited by the tyrosine kinase inhibitor herbimycin A (Lepple-Wienhues et al., 1998, 2000), whereas the release of taurine from swollen Jurkat lymphocytes is unaffected by these modulations (Lang et al., 2000a). Moreover, in recent years, evidence has been presented that indicates existence of several pathways in mediation of the loss of various organic osmolytes, e.g., in astrocytes, the presence of Hg^{2+} inhibits the swelling-activated taurine release, while the release of *myo*-inositol is increased (Aschner et al., 1998). The release of taurine can also be distinguished from the release of creatine by the pharmacological inhibitor profile, time of inactivation of the leak pathways and sensitivity towards changes in cell volume (Bothwell, Styles & Bhakoo, 2002). Furthermore, the release of *myo*-inositol upon cell swelling seems to be composed of a two-component system (Isaacks et al., 1999), and application of modulators of tyrosine kinase activity reveals that two separate anion channels mediate the loss of taurine (Mongin et al., 1999). These studies indicate the existence of numerous pathways for conducting the volume-sensitive release of both inorganic and organic osmolytes in mammalian cells.

The role of calcium in the regulation of the activity of the swelling-induced osmolyte channels and the overall RVD response is controversial. Whether cells show (i) a change in the intracellular Ca^{2+}-concentration ($[Ca^{2+}]_i$) upon cell swelling, (ii) an extracellular Ca^{2+}-dependency of the RVD response or activity of the respective osmolyte channels, or (iii) a Ca^{2+}-mediated regulation of the activity of the involved signaling proteins is clearly dependent on the cell type in question (McCarty & O'Nell, 1992; Foskett, 1994). In general, in those cell types where cell swelling does not result in any detectable change in $[Ca^{2+}]_i$, e.g., in C6 glioma cells, H4IIE hepatoma cells, Ehrlich ascites tumor cells, neurons, NIH3T3 fibroblast cells (Strange et al., 1993; Schliess, Schreiber & Haussinger, 1995; Leaney, Marsh & Brown, 1997; Jørgensen et al., 1997; Herring et al., 1998; Pedersen et al., 2002), no Ca^{2+}-dependency of either the RVD response or the activity of the respective osmolyte-conducting leak pathways can be demonstrated. On the other hand, in those cell types where modulations of $[Ca^{2+}]_i$ and extracellular Ca^{2+} regulate the RVD response, e.g., intestine epithelial cells, osteoblast-like cells, renal TALH cells, the observed Ca^{2+} requirement is often associated with activity of volume-sensitive, Ca^{2+}-activated K^+ channels (MacLeod and Hamilton, 1999; Pasantes-Morales & Morales-Mulia, 2000; Weskamp, Seidl & Grissmer, 2000; Tinel, Kinne-Saffran & Kinne, 2002). The activity of $I_{Cl,vol}$ is generally not affected by modulations of internal or external Ca^{2+} levels (Leaney et al., 1997; Nilius et al., 1997; Sakai, Nakamura & Kuno, 1999), but has in some cases been shown to require a minimum level of $[Ca^{2+}]_i$ (Szucs et al., 1996; Shen et al., 2001). Finally, in some cell types, changes in $[Ca^{2+}]_i$ seem to be an epiphenomenon upon cell swelling and not related to the RVD response (Foskett et al., 1994; Moran et al., 1997b; Altamirano, Brodwick & Alvarez-Leefmans, 1998; Morales-Mulia et al., 1998; Sheader, Brown & Best, 2001). More recently it has been demonstrated that Ca^{2+} modulates the swelling-induced taurine efflux from HeLa cells and it was suggested that HeLa cells require Ca^{2+} influx and Ca^{2+} mobilization from intracellular, thapsigargin-sensitive stores in order to maintain a sustained volume-sensitive taurine release (Olivero & Stutzin, 2004).

The purpose of the present study was to investigate the role of Ca^{2+}-regulated proteins in the intracellular signaling cascade, which is activated by hypotonic cell swelling, and which leads to activation of volume-sensitive taurine-permeable leak pathways in HeLa cells. Our results indicate that despite the

lack of a detectable global rise in $[Ca^{2+}]_i$ upon cell swelling and independence of extracellular Ca^{2+} of the taurine-release pathway, Ca^{2+}-activated proteins such as calmodulin (CaM) and phorbol ester-sensitive protein kinase C (PKC) isoforms are involved in the regulation of the activity of the volume-sensitive taurine-release pathway in HeLa cells.

Materials and Methods

Cell Culture

The human cervical cervix carcinoma cell line, HeLa, was maintained as a monolayer culture in Earle's minimum essential medium supplemented with 10% v/v newborn calf serum, 2 mM glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin, at 37°C, 5% CO_2, 95% humidity. Cells were passaged twice weekly (1:8 ratio) in 0.25% trypsin-containing phosphate-buffered saline containing in mM: 137 NaCl, 2.6 KCl, 6.5 Na_2HPO_4, 1.5 KH_2PO_4.

Inorganic Media

Isotonic NaCl medium contained in mM: 150 NaCl, 1 Na_2HPO_4, 1 $CaCl_2$, and 10 *N*-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES). The Ca^{2+}-free NaCl medium had no added $CaCl_2$, while the other components remained unchanged. In some cases, the Ca^{2+}-free NaCl medium was supplemented with 1.5 mM ethylene-glycol-bis-β-amino-ethyl-(ether)N,N,N′,N′-tetra acetic acid (EGTA). Isotonic KCl medium contained in mM: 150 KCl, 1.3 $CaCl_2$, 0.5 $MgCl_2$, and 10 HEPES. Hypotonic media were obtained by reduction of the NaCl or the KCl to 93 mM and 95 mM, respectively, while the other components remained unchanged. pH was in all solutions adjusted at 7.4. The osmolarity of the isotonic and hypotonic solutions was estimated at 295 and 195 mosmole per liter, respectively (Halbmikro-Osmometer, Knaur). Solutions containing high K^+ concentrations were used in order to eliminate the outward transmembrane K^+ gradient, i.e., the driving force for RVD, and thereby prolonging the cell swelling-activated response in hypotonic media (Kirk & Kirk, 1993).

Chemicals

Penicillin, streptomycin, glutamine and Earle's minimum essential medium were purchased from Life Technologies (Denmark). Newborn-calf serum was purchased from Life Technologies (Denmark) and Biological Industries (Israel). The concentration and vehicle of the stock solutions are given in parentheses. [^{14}C]-taurine was from Perkin Elmer Life Sciences (Boston, MA). Fura-2-AM (DMSO, 10 mM) was purchased from Molecular Probes (Netherlands), whereas histamine (ddH_2O, 5 mM/100 mM), bradykinin (ddH_2O, 1 mM), EGTA (Tris, pH 7.4), 4.4′-diisothiocyano-2.2′-stilbenedisulfonic acid (DIDS, ddH_2O, 20 mM), phorbol 12-myristate 13 acetate (PMA, 0.1 mM / 1 mM), pyrilamine (ddH_2O,100 mM), N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W-7, ddH_2O, 5 mM), 4′-methoxybenzenesulfonyl)amino-N-(4′-chlorophenyl)-2-propyl-N-methylbenzylamine phosphate (KN-62, DMSO, 10 mM), 1-(5-iodonaphthalene-1-sulfonyl)-1H-hexahydro-1, 4-diazepine hydrochloride (ML-7, 50% ethanol, 5 mM) and cyclosporine A (CsA, 96% ethanol, 1 mM) were purchased from Sigma (St. Louis, MO). Gö6976 (DMSO, 500 μg/ml) and Gö6850 (DMSO, 1 mg/ml) were purchased from Calbiochem (Bie & Berntsen, Denmark).

Estimation of the Rate Constant for Taurine Efflux

Taurine efflux measurement and estimation of the rate constant were performed at room temperature (RT, 20–22°C) as described previously (Kirk & Kirk, 1993; Lambert & Falktoft, 2000). In brief, cells were seeded 24 h prior to experiments on 35-mm diameter polyethylene dishes. At the day of experiments confluence was 80–90% and cells were preloaded with [^{14}C]-taurine (70–143 nCi/ml) for 2 h at 37°C in either growth medium or in 5 mM glucose-containing NaCl medium (serum starvation). Prior to initiation of the efflux experiments, the incubation solution was aspirated and cells were washed five times by removal and addition of 1 ml isosmotic NaCl medium to remove excess extracellular [^{14}C]-taurine. One milliliter of isosmotic solution was added to the dish after removal of the final wash medium, i.e., the total amount of medium in the dish during the experiment is 1 ml. In some cases, cells were pretreated with compounds prior to initiation of taurine efflux measurements by adding 1 ml of experimental solution to the dish and allowing incubation for the desired amount of time. Efflux measurements were performed at 2 min time intervals by transferring all the experimental medium from the dish to a scintillation vial for estimation of ^{14}C activity (β-scintillation counting, Ultima Gold) and replacing the sample by another 1 ml of experimental solution. The amount of [^{14}C]-taurine remaining inside the cells at the end of the efflux experiment was estimated by treating the cells with 0.5 N NaOH for 1 h, washing the dish twice with distilled water, and measuring the ^{14}C activity in the NaOH as well as in the water washouts. The total intracellular ^{14}C activity was estimated as the sum of the ^{14}C activity released during the release experiment and the ^{14}C activity remaining inside the cells at the end of the efflux experiment. The natural logarithm to the fraction of ^{14}C activity remaining intracellularly at a given time (t) was estimated and plotted versus time (*see* Fig. 1*A*). The rate constant for the taurine efflux at time point t was estimated as the negative slope of the graph between time point t and its proceeding time point.

Measurement of the Free, Intracellular Calcium Concentration ($[Ca^{2+}]_i$)

Cells were seeded 24 h prior to experiments on rectangular (10 × 50-mm, Menzel-Gläser), HCl- and ethanol-washed cover slips. At the day of the experiments confluence was 70–90%. Changes in the free intracellular calcium concentration ($[Ca^{2+}]_i$) were assessed by preloading the cells with the calcium-sensitive fluorescent probe, fura-2-AM (2.5 μM) for 45–60 min at room temperature in isotonic 5 mM glucose-containing NaCl medium (*see* Inorganic media). Subsequently the coverslip was mounted on a custom-made holder and placed in a magnetic-stirred cuvette of a PTI RatioMaster spectrophotometer with a 75 W Xenon arc lamp. Following a 10 min washing period of the cells, i.e., perfusion of the cuvette with isotonic solution, excitation was set at 345 nm (or in some cases 350 nm) and 390 nm and emission was measured at 510 nm. Data are evaluated as the 345/390 nm or the 350/390 nm ratio.

Estimation of Changes in Cell Volume, Light Scatter Measurements

Large-angle light scattering was used as an estimation of relative changes in cell volume. Cells were seeded on cover slips as described for estimation of $[Ca^{2+}]_i$. On the day of experiments, cells were washed once with isotonic NaCl medium, and the cover slip was mounted on the holder, placed at a 50-degree angle relative to the excitation light, in a magnetic-stirred cuvette of the PTI RatioMaster spectrophotometer. Following a 10 min washing period,

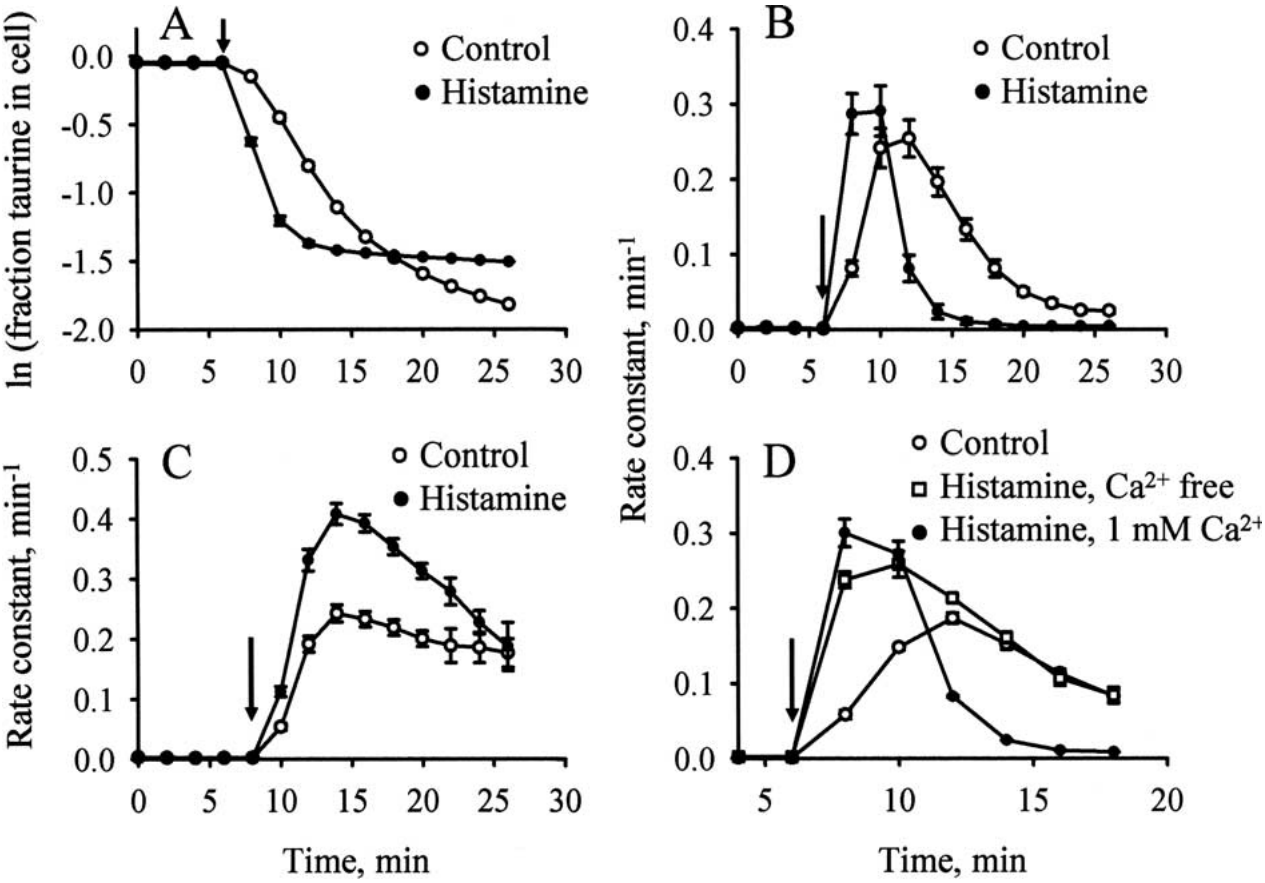

Fig. 1. Effect of Ca^{2+} on the swelling-induced taurine-release. Cells were grown to 80% confluence and preloaded with [^{14}C]-taurine for 2 hours. The efflux experiments were performed in NaCl or KCl media, and the rate constants for the taurine efflux, estimated as described in Materials and Methods, are given as mean values ± SEM. The osmolarity of the extracellular medium was reduced to 2/3 of the isotonic value at the time indicated by the arrow. (*A*) Experiments were performed in isotonic/hypotonic NaCl medium. The natural logarithm of the fraction of ^{14}C-taurine remaining in the cells was estimated at 2 min intervals and plotted versus time. The medium was shifted from isotonic NaCl medium to hypotonic medium at the time indicated by the arrow. Histamine (10 μM, *filled symbols*) was present throughout the hypotonic exposure. (*B*) The rate constant for the taurine efflux at a given time point was estimated from the negative slope between the time point and its proceeding time point in panel *A*. Data points are based on n = 3–17 independent experiments. (*C*) Experiments were performed in isotonic/hypotonic KCl medium. Data points are based on n = 3–16 independent experiments. (*D*) Experiments were performed in isotonic and hypotonic NaCl medium containing either 1 mM Ca^{2+} with histamine (*filled circles*), without histamine (*empty circles*), or 0 mM Ca^{2+} supplemented with 1.5 mM EGTA and histamine (*empty squares*). Data points are based on 2–3 sets of independent experiments.

i.e., continual perfusion of the cuvette with isotonic solution at a flow rate of 0.7 ml min^{-1}, light scatter measurements were initiated by keeping the excitation wavelength at 577 nm and the emission at 580 nm. Cells were exposed to a rapid change in extracellular osmolarity by application of a brief (ca. 200 s) increase in flow rate of the perfusion system to 3.5 ml min^{-1}. As the collected emitted light is inversely correlated with cell volume, data are given as the inverse of the 580 nm emission relative to a stable 580 nm emission intensity for a 200 s period under isotonic conditions.

Statistical Analysis

Data are shown as means ± standard error of mean (SEM) or as individual experiments representative of a minimum of three independent experiments. Student's t-test (two-tailed, paired or homoscedastic, as appropriate) was applied to evaluate statistical significance and $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Ca^{2+}-Mobilizing Hormones Potentiate the Swelling-induced Taurine Loss from HeLa Cells

Previous studies in various cell systems have shown that addition of hormones, which mobilizes intracellular Ca^{2+} and induces Ca^{2+}-influx across the cell membrane at the time of a hypotonic challenge, results in an increase of the swelling-induced release of organic osmolytes, e.g., taurine, and in acceleration of the RVD-response (Lambert, 2004). Similarly, it has been shown, addition of micromolar concentrations of the Ca^{2+}-mobilizing agents ATP or UTP to HeLa cells at the time of hypotonic exposure results in an increase in the rate constant

for the taurine efflux (Lambert & Sepulveda, 2000). This is confirmed in Fig. 1*A* and 1*B*, where it is shown that exposure to hypotonic media results in a transient increase in the rate constant for the taurine efflux and that addition of histamine (10 μM), which is a well-described Ca^{2+}-mobilizing agonist in HeLa cells (Bristow & Zamani, 1993), at the time of hypotonic exposure results in a significant 1.32 ± 0.06-fold ($n = 17$, $P \ll 0.001$, paired *t*-test) increase in the maximal rate constant for the swelling-induced taurine efflux. Similarly, addition of the Ca^{2+} agonist bradykinin (1 μM) results in a significant 1.31 ± 0.04-fold increase in the maximal rate constant ($n = 10$, $P = 0.004$, paired *t*-test). Furthermore, inclusion of histamine (Fig. 1*B*) or bradykinin (*data not shown*) in the hypotonic medium also results in a subsequent faster inactivation of the taurine efflux following hypotonic exposure.

In order to examine whether the Ca^{2+} agonist-induced inactivation of the taurine-release pathway in RVD-performing HeLa cells was due to either an inhibition of the volume-sensitive taurine efflux pathway or caused by an accelerated RVD, we added histamine to cells in a hypotonic high K^+ medium, which clamps the K^+ gradient across the cell membrane and hence the driving force for RVD and prevents the cells from reducing their cell volume after the initial cell swelling. Figure 1*C* shows that the presence of histamine (10 μM) in the hypotonic KCl medium potentiates the rate constant for the swelling-induced taurine efflux, and it is estimated that the maximal rate constant is increased 1.75 ± 0.05-fold ($n = 17$, $P \ll 0.001$) in the presence of histamine. Similar to the above findings for cells in NaCl media, the actions of histamine on the swelling-induced taurine release pathway in HeLa cells in hypotonic KCl medium is mimicked by the Ca^{2+}-mobilizing agonists bradykinin (potentiation = 1.27 ± 0.10-fold, 1 μM, $n = 6$, $P = 0.02$), thrombin (potentiation = 1.94 ± 0.08-fold, 5 IU/ml, $n = 14$, $P \ll 0.001$), ATP (potentiation = 1.47 ± 0.04-fold, 5 μM, $n = 11$, $P \ll 0.001$), and UTP (potentiation = 1.38 ± 0.07-fold, 5 μM, $n = 3$, $P = 0.02$). However, as seen from Fig. 1*C*, the presence of histamine in the hypotonic KCl medium does not accelerate the inactivation of the taurine-release pathway following hypotonic exposure, as observed for HeLa cells exposed to histamine in a hypotonic NaCl medium (*see* Fig. 1*B*). These results indicate that the observed faster reduction in the rate constants for the taurine loss from cells in Ca^{2+} agonist-containing hypotonic NaCl media is a consequence of an accelerated RVD and is not caused by Ca^{2+}-mediated inhibition of the taurine release pathway in HeLa cells.

The Ca^{2+}-stimulated Taurine Loss from Hypotonically Swollen HeLa Cells Is Mediated by the Volume-sensitive Taurine Efflux Pathway

The predicted molecular structure of proteins known to conduct Cl^- movement across cell membranes apparently indicates channel pores of a diameter of 4.5–7 Å, e.g., 5.3 Å for the cystic fibrosis transmembrane regulator/cAMP-activated Cl^--channel (CFTR) (Illek et al., 1999), 4.5–6 Å for ClC-channels (Fahlke, 2001), and 6–7 Å for Ca^{2+}-activated Cl^--channels (Cl(Ca)C's) (Qu & Hartzell, 2000). Hence, they are likely also to be permeable to organic osmolytes such as taurine (diameter ≈ 6.4 Å; Hall et al., 1996). That Cl^--conducting pathways are permeable to taurine is supported by electrophysiological- and tracer technique-based findings for $I_{Cl,vol}$ / VSOAC (Banderali & Roy, 1992; Roy, 1995; Jackson et al., 1996), voltage-dependent anion channels (VDAC/porin) (Steinacker et al., 1997; Thinnes et al., 2000) and outwardly-rectifying Cl^--channel, ORCC (Thinnes et al., 2001). Thus, the Ca^{2+} agonist-induced increase in the rate constant for the swelling-induced taurine efflux could possibly be due to Ca^{2+}-mediated stimulation of the Ca^{2+}-activated channels for K^+ and Cl^-. As the activity of Ca^{2+}-activated channels for K^+ and Cl^- in HeLa cells is dependent upon the presence of extracellular Ca^{2+} (Sauve et al., 1990, 1991; Russell, McPherson & Dormer, 1995), we examined the dependence on extracellular Ca^{2+} of the histamine-augmented swelling-induced taurine efflux from HeLa cells. Figure 1*D* shows that replacement of extracellular Ca^{2+} with 1.5 mM EGTA causes a minor reduction in the histamine-potentiated maximal rate constant for the hypotonic-induced taurine efflux from HeLa cells. Similar data were obtained after application of bradykinin, i.e., the maximal rate constant for bradykinin (1 μM)-treated cells was reduced to 88 ± 6% ($n = 7$) following removal of extracellular Ca^{2+}. This indicates that addition of Ca^{2+}-agonists, although to a lesser extent, increases the maximal rate constant for the swelling-induced taurine efflux by a pathway essentially independent of extracellular Ca^{2+}. On the other hand, Fig. 1*D* furthermore shows that the Ca^{2+} agonist-induced faster inactivation of the hypotonic-induced taurine efflux is completely dependent on the presence of extracellular Ca^{2+}. These data taken together indicate that the Ca^{2+} agonist-induced augmentation of the maximal rate constant for the taurine efflux does not reflect an increased activity of the Ca^{2+}-activated ion channels.

Provided that the volume-sensitive taurine efflux pathway is mediating the Ca^{2+}-augmented taurine loss from swollen HeLa cells, we would expect that the increased taurine loss in the presence and absence of Ca^{2+}-mobilizing agonists is equally sensitive to-

wards well-known inhibitors of the volume-sensitive taurine efflux pathway. The IC_{50} for DIDS inhibition of the taurine efflux was estimated at 0.98 μM in KCl medium and 1.15 μM in NaCl medium in the absence of histamine and at 0.78 μM in KCl medium and 2.5 μM in NaCl medium in the presence of histamine. The IC_{50} for the arachidonic acid-mediated inhibition of the taurine efflux in NaCl medium was estimated at 6.8 μM and 6.7 μM in the absence and presence of histamine. Moreover, we were not able to detect any difference in the sensitivity of the hypotonic-induced taurine efflux towards changes in the extracellular osmolarity (range: 300 to 200 mOsmole/l) in the absence or presence of histamine, i.e., normalized maximal rate constants for the taurine efflux at a given extracellular osmolarity in the absence and presence of histamine, respectively, were not significantly different ($P = 0.82$ in NaCl medium and $P = 0.23$ in KCl medium, *data not shown*). As both the basic and histamine-induced potentiated swelling-induced taurine efflux are equally affected by the two common inhibitors of the swelling-induced taurine-release pathway, DIDS and arachidonic acid, and as the sensitivity of the taurine efflux towards changes in cell volume is similar in the absence and presence of histamine, we suggest that addition of Ca^{2+}-mobilizing agonists concomitant with a hypotonic shock results in an increased activity of the swelling-induced taurine release pathway in HeLa cells. In contrast, the Ca^{2+} agonist-induced faster inactivation of the taurine leak pathway is dependent on extracellular Ca^{2+} (*see* Fig. 1*D*) and therefore probably reflects stimulation of Ca^{2+}-activated K^+- and Cl^--channels, leading to a faster loss of intracellular KCl and thereby acceleration of the RVD. To confirm that RVD is, indeed, accelerated in the presence of histamine, we applied the light-scattering-technique for measurement of relative cell volume changes. Figure 2 shows that after a shift from extracellular isotonicity to hypotonicity, application of histamine (20 μM) at the time of maximal cell swelling results in an accelerated restoration of the cell volume. We suggest that addition of Ca^{2+}-mobilizing agonists at the time of hypotonic exposure results in (i) an augmentation of the swelling-induced taurine efflux through increased activity of the volume-sensitive leak pathway and (ii) a faster inactivation of the volume-sensitive taurine efflux pathway most probably due to activation of Ca^{2+}-activated K^+ and Cl^- channels and a subsequently accelerated RVD.

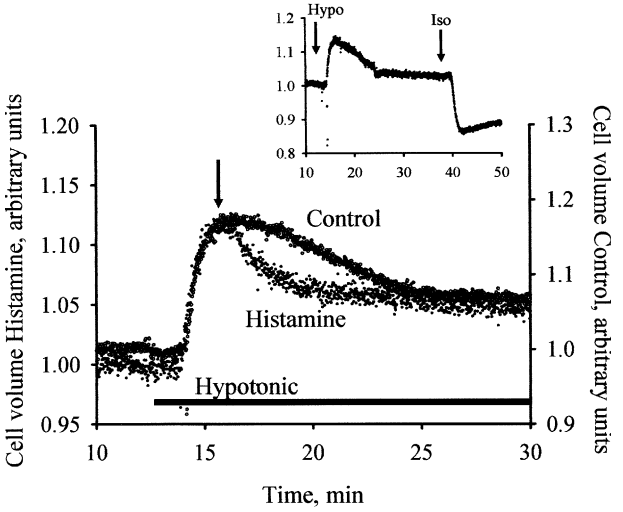

Fig. 2. Addition of a Ca^{2+} agonist upon cell swelling accelerates the regulatory volume decrease. Large-angle light scattering was performed to monitor changes in relative cell volume, as described in Materials and Methods. Adherent cells were allowed to acclimate in isotonic medium by perfusing the cells with isotonic medium until a stable signal was obtained. Extracellular hypotonicity was induced at the time indicated by the bar by rapidly changing the isotonic cuvette solution to a $\frac{2}{3}$ hypotonic solution. Upon maximal cell swelling, perfusion was terminated and control cells were subsequently allowed to volume-regulate in hypotonic NaCl medium, whereas *Histamine* denotes the addition of histamine (20 μM) directly into the cuvette upon maximal cell swelling. The scales on the ordinate are different in order to align the peak volume values. The experiment shown is representative of 4 independent experiments. The insert in Fig. 2 shows the relative volume changes in control cells after perfusion with isotonic solution and shift to hypotonicity (*first arrow*), followed by a subsequent RVD response and re-exposure to isotonic solution (*second arrow*). Provided that the cells perform RVD and lose osmolytes when surrounded by hypotonic NaCl medium, a restoration of extracellular osmolarity to isotonicity is perceived by the cells as hypertonicity, thereby resulting in cell shrinkage compared to the initial cell volume. We applied the 'iso/hypo/iso'-protocol in each experimental setup as a control, i.e., light scattering actually reflected changes in cell volume. The experiment shown is representative of at least 10 independent experiments.

Exposure to Ca^{2+}-agonists Prior to a Hypotonic Challenge Potentiates the Activity of the Volume-sensitive Taurine Leak Pathway in HeLa Cells

The increased taurine release in the presence of Ca^{2+} agonist could reflect (i) a "direct" activation of the volume-sensitive taurine leak pathway or (ii) an "indirect" effect caused by increased activity of Ca^{2+}-sensitive proteins involved in the regulation of the volume-sensitive taurine leak pathway. Provided the Ca^{2+}-agonist-induced augmentation of the taurine release reflects an "indirect" effect, one would expect that a transient increase in $[Ca^{2+}]_i$ prior to a hypotonic exposure would 'prime' Ca^{2+}-sensitive proteins and still augment the taurine release in a subsequent hypotonic challenge even though $[Ca^{2+}]_i$ would have returned to the resting level. On the other hand, if the Ca^{2+} agonist-induced increase in taurine release from swollen cells is a consequence of a "direct" interaction between Ca^{2+} and the swelling-induced taurine release pathway, we would not expect a Ca^{2+} agonist-induced augmentation of the taurine

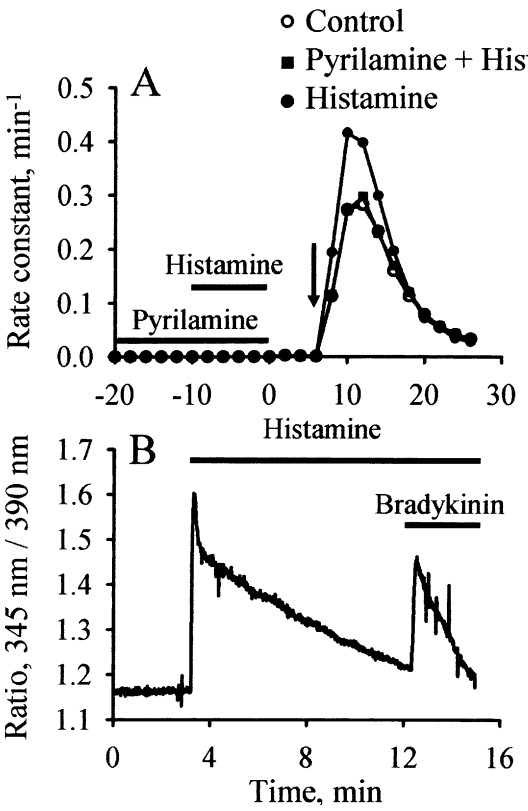

Fig. 3. The Ca^{2+}-mediated potentiation of the volume-sensitive taurine efflux is not an effect of Ca^{2+} *per se* on the taurine transporting system. (*A*) Cells, grown to 80% confluence, were loaded with [^{14}C]-taurine for 2 hours and washed with NaCl medium. Cells were pre-exposed to isotonic NaCl medium (20 min, *empty circles*), histamine (10 μM; 10 min, *filled circles*), or pyrilamine (1 μM; 20 min) plus histamine (10 μM; 10 min, *filled squares*) as indicated by the bars. Taurine efflux experiments were initiated at time zero and performed in NaCl medium in the absence of histamine and pyrilamine and with a shift in the extracellular osmolarity to $\frac{2}{3}$ of the isotonic value at the time indicated by the arrow. Rate constants for the taurine efflux were estimated as described in Materials and Methods. The experiment shown is representative of 4 independent experiments. Experimental mean values are summarized in Table 1. (*B*) Cells were loaded with fura-2-AM as described in Materials and Methods. The data show the ratio of the fluorescence intensities following excitation at 345 nm and 390 nm, respectively, and collection of the emission light at 510 nm. Ratio measurements were performed in isotonic NaCl medium. Bars indicate the presence of histamine (10 μM) and bradykinin (1 μM). The data shown are representative of 6 independent experiments.

release from cells in which $[Ca^{2+}]_i$ has returned to resting values. Hence, we applied an experimental protocol in which cells were exposed to Ca^{2+} agonists before introduction of hypotonic conditions. From Fig. 3*A* it is seen that pre-incubation with histamine (10 μM, 10 min) under isotonic conditions still potentiates the activity of the swelling-induced taurine efflux pathway in the subsequent hypotonic challenge. In 24 experiments we estimate that the maximal rate constant for the swelling-induced taurine efflux is significantly increased 1.45 ± 0.07 fold ($P < 0.001$) by histamine-preexposure (Table 1). Figure 3*B* shows that addition of histamine and bradykinin results in transient increases in $[Ca^{2+}]_i$, and that $[Ca^{2+}]_i$ returns to unstimulated values within 8–10 min even in the continual presence of the hormones. It is further noted that upon complete removal of the Ca^{2+}-agonists from the extracellular medium, $[Ca^{2+}]_i$ immediately returns to its resting value (*data not shown*). Pre-exposure to bradykinin also results in a significant augmentation of the maximal rate constant for the taurine efflux by 1.31 ± 0.04 fold ($n = 10$, $P < 0.001$) during a subsequent hypotonic exposure (Table 1). HeLa cells express H_1-receptors, not H_2-, H_3- or H_4-receptors (Hazama, Yada & Okada, 1985; Bristow et al., 1993), and the presence of the selective H_1-receptor antagonist pyrilamine (1 μM) (Hill et al., 1997) completely abolishes the histamine-induced augmentation of the maximal rate constant for the swelling-induced taurine efflux (Fig. 3*A* and Table 1). Exposing HeLa cells to pyrilamine has no effect on the swelling-induced taurine efflux in itself nor does the presence of pyrilamine affect the bradykinin-mediated potentiation of the swelling-induced taurine release (Table 1). Thus, the effect of histamine is specific and mediated through the interaction of H_1-receptors.

Stimulation of Ca^{2+}-activated channels for K^+ and Cl^- in HeLa cells requires the presence of extracellular Ca^{2+} (Sauve et al., 1990, 1991; Russell et al., 1995) and to verify that the increased taurine release from hypotonically swollen HeLa cells after pre-exposure to histamine is a consequence of increased activity of the volume-sensitive taurine release pathway, and not mediated by Ca^{2+}-activated ion channels, we pre-exposed the HeLa cells to histamine in a Ca^{2+}-depleted medium and performed the subsequent efflux experiments in the presence of Ca^{2+}. From Table 1 it is seen that histamine preexposure in Ca^{2+}-free media still results in augmentation of the taurine release of 1.36 ± 0.18-fold ($n = 3$) during the subsequent hypotonic exposure. In Ca^{2+}-free + EGTA-medium the relative taurine release from hypotonically swollen HeLa cells is estimated at 1.20 ± 0.07-fold after pre-incubation with histamine (*see* Table 1), which is significantly different from control cells ($n = 6$, $P = 0.02$). It therefore seems reasonable to exclude a contribution of Ca^{2+}-activated ion channels in mediating the increased taurine efflux. It is noted that incubation of the cells in a Ca^{2+}-free or Ca^{2+}-free plus EGTA-containing medium in itself has a stimulatory effect on the subsequently swelling-induced taurine release (*see* Table 1). The underlying mechanism was not investigated in this study but a possible explanation is that removal of extracellular Ca^{2+}, which often leads to a minor reduction in $[Ca^{2+}]_i$, activates signals for replenishment of intracellular Ca^{2+} stores, thereby

Table 1. The potentiating effect of pre-exposure to Ca^{2+}-mobilizing agonists on the swelling-induced taurine efflux are partially independent of extracellular Ca^{2+}

		Control	Histamine, 10 μM	Bradykinin, 1 μM
Ca^{2+}, 1.3 mM		1	1.45 ± 0.07 (n = 24)[c]	1.31 ± 0.04 (n = 10)[c]
Ca^{2+}, 1.3 mM	Pyrilamine, 1 μM	1.01 ± 0.02 (n = 4)[d]	1.03 ± 0.05 (n = 4)[d]	1.37 ± 0.04 (n = 5)[c,e]
Ca^{2+}, 1.3 mM	DIDS 10 μM	0.50 ± 0.12 (n = 5)[a]	0.52 ± 0.09 (n = 5)[b]	—
	100 μM	0.21 ± 0.03 (n = 5)[a]	0.23 ± 0.04 (n = 5)[b]	—
Ca^{2+} free		1.27 ± 0.23 (n = 3)[h]	1.36 ± 0.18 (n = 3)[f]	—
Ca^{2+} free + EGTA		1.30 ± 0.18 (n = 6)[h]	1.20 ± 0.07 (n = 6)[g]	—

Cells, grown to 80% confluence, were preloaded with [^{14}C]-taurine for 2 h and prepared for efflux experiments as described in Materials and Methods. Prior to initiation of taurine efflux measurements, the cells were incubated with compounds as listed in the Table. Column "Control" indicates cells incubated for 20 min in isotonic NaCl medium containing either 1.3 mM Ca^{2+}, 0 mM Ca^{2+} ("Ca^{2+} free") or 0 mM Ca^{2+} supplemented with 1.5 mM EGTA ("Ca^{2+} free + EGTA"). Columns "Histamine" and "Bradykinin" indicate cells pre-exposed to histamine (10 min) and bradykinin (10 min), respectively, whereas "Pyrilamine" denotes cells pre-exposed for 10 min to pyrilamine followed by a further addition of histamine (10 μM; 10 min) or bradykinin (1 μM; 10 min) (as shown in Fig. 3*A*). Efflux experiments were subsequently performed in isotonic and hypotonic NaCl medium with no added compounds, with the exception of column "DIDS", which indicates the presence of DIDS during the efflux experiments. The rate constants were estimated as described in Materials and Methods, and the maximal rate constant for the swelling-induced taurine efflux (corresponding to time 10 min in Fig. 3*A*) was related to the appropriate control. We note that the obtained values for the taurine-release after incubation with histamine in a "Ca^{2+}-free" or "Ca^{2+}-free + EGTA" medium are given relative to the values obtained in "Ca^{2+}-free-" and "Ca^{2+}-free + EGTA"-medium, respectively, whereas the taurine release obtained from cells after exposure to Ca^{2+}-depleted media is related to the control. Data are given as means ± SEM. The number in brackets indicates the number of independent experiments. All statistical tests are performed on absolute values with the exception of b.

[a] $P < 0.01$; values tested against control cells.

[b] $P < 0.17$ (10 μM) and $P < 0.72$ (100 μM); the relative effect of DIDS on control cells without histamine is tested against the effect of DIDS in histamine-treated cells.

[c] $P < 0.01$, values tested against control cells.

[d] $P = 0.35$–0.50; values tested against control cells.

[e] $P = 0.43$; values for pyrilamine plus bradykinin-treated cells are tested against bradykinin-treated cells.

[f] $P = 0.16$; values for cells in Ca^{2+}-free medium are tested against control cells in Ca^{2+}-containing medium.

[g] $P = 0.02$, the effect of histamine is tested for cells in Ca^{2+}-free + EGTA medium.

[h] $P = 0.42$–0.71; values are tested against cells in Ca^{2+}-containing medium.

causing an increase in $[Ca^{2+}]_i$ upon re-addition of extracellular Ca^{2+} at initiation of the efflux experiments. This would increase the rate constants for the swelling-induced taurine release (*see* Fig. 1*B*).

From Table 1 it is also seen that the maximal rate constant for the swelling-induced taurine efflux from control cells and cells pre-exposed to histamine is reduced by DIDS to the same degree (values not significantly different between control and histamine-treated cells, $P = 0.17$ at 10 μM DIDS and $P = 0.72$ at 100 μM DIDS). Thus, the volume-sensitive taurine release pathway is mediating the increased taurine loss after short-term pre-exposure to histamine and in accordance with the results obtained for the increased taurine loss observed after addition of histamine at the time of hypotonic exposure (*see* above).

To further verify that the effect of Ca^{2+} on the activity of the taurine leak pathway is mediated by signaling proteins and does not reflect a direct activation of the taurine leak pathway, we took advantage of the fact that the activity of the volume-sensitive taurine efflux pathway is reduced following two hours of serum starvation (Lambert & Falktoft, 2000). From Fig. 4*A* it is seen that there is a small and slight increase with time in the rate constant for the taurine efflux from HeLa cells submerged in isotonic KCl solution. In 16 experiments it is estimated that the rate constants increase 1.5 ± 0.1-fold within 10 min (Fig. 4*B*), probably due to high K^+-induced isosmotic cell swelling (Ikehara et al., 1991). It is noted that the rate constants for the taurine release from cells under isotonic conditions is approximately 100–200 times less than the estimated values obtained from cells under hypotonic conditions (compare, e.g., Fig. 1*C* and Fig. 4*A*). The putative isosmotic cell swelling apparently activates the volume-sensitive taurine leak pathway, as the rate constant for the taurine efflux is reduced to 62 ± 8% and 56 ± 8% of the control in the presence of 5 μM and 10 μM DIDS, respectively ($n = 4$ for each concentration, $P < 0.05$). Addition of histamine (10 μM) to HeLa cells submerged in the isotonic KCl medium results in a rapid significant augmentation of the rate constants for the taurine efflux (Fig. 4*A* and Fig. 4*B*, $n = 8$, $P = 0.003$), which implies that an increase in $[Ca^{2+}]_i$ also augments the activity of the taurine leak pathway in cells under isotonic conditions. Analogous to the findings in hypotonically swollen cells, it is seen from Fig. 4*B* that the effect of histamine is mimicked by application of other Ca^{2+}-agonists, e.g., addition of the Ca^{2+}-ionophore ionomycin (250–1000 nM) to cells in isotonic KCl medium results in a significant increase in the relative rate constant for the taurine efflux of 2.2 ± 0.2-fold ($n = 7$, $P = 0.025$), which is

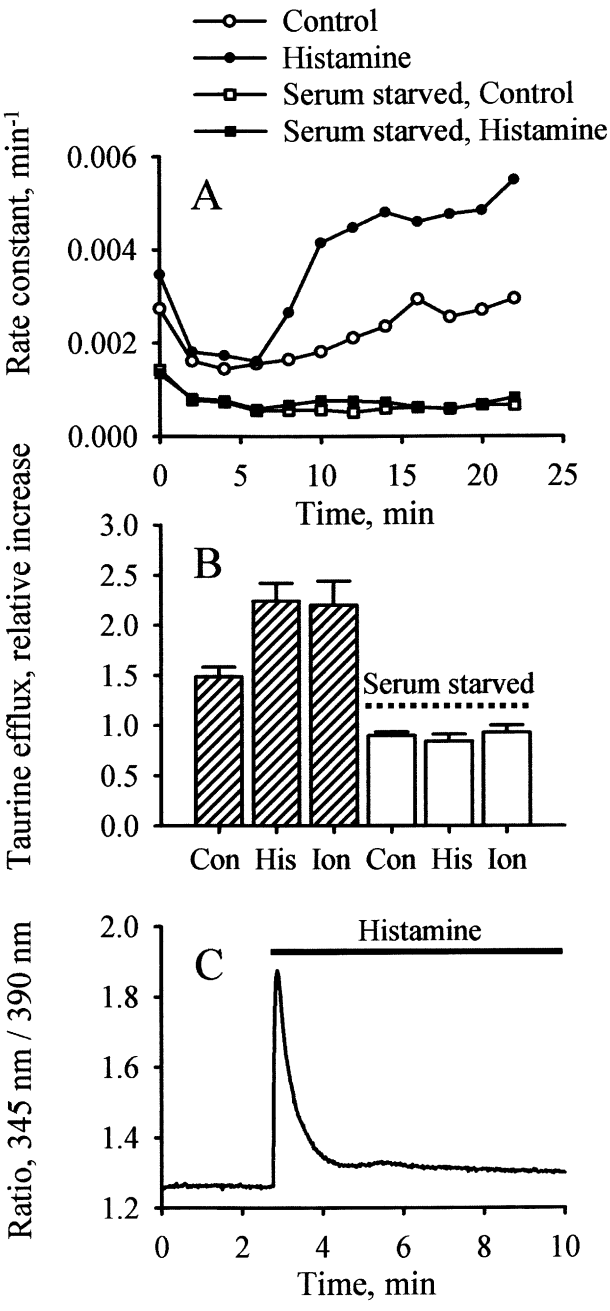

Fig. 4. Serum starvation prevents Ca^{2+}-agonist augmentation of the taurine efflux from cells under isotonic conditions. (*A-B*) Cells were grown to 80% confluence and preloaded with [^{14}C]-taurine for 2 hours in either growth medium (Panel *A*, *circles*; Panel *B*, *hatched bars*) or NaCl medium containing 5 mM glucose (Panel *A*, *squares*; Panel *B*, *white bars*) as described in Materials and Methods. Efflux experiments were performed in isotonic KCl medium, and rate constants for taurine efflux estimated as described in Materials and Methods. (*A*) Histamine (10 μM, *filled symbols*) was included from time 6 min. The experiment shown is representative of 6–8 independent experiments. *See* Fig. 4*B* for values. (*B*) Experiments were performed as shown in (*A*) and from time 6 min in the presence of histamine (10 μM) and ionomycin (250–1000 nM), respectively. In each experimental situation a mean efflux rate constant was calculated based on 3–4 data points both prior to and after addition of compounds. The relative increases in efflux rate constants are given as means ± SEM. The number of experiments was 16/19 ("Con" ± serum starved), 8/6 ("His" ± serum starved) and 7/7 ("Ion" ± serum starved). We note that addition of 250–1000 nM ionomycin resulted in a quantitatively similar relative increase in taurine loss from HeLa cells, hence the mean values represent the effect of ionomycin in the concentration range 250–1000 nM. (*C*) Cells were loaded with fura-2-AM (2.5 μM) for 2 h at room temperature in NaCl medium containing 5 mM glucose as described in Materials and Methods. The data show the ratio of the fluorescence intensities following excitation at 345 nm and 390 nm, respectively, and collection of the emission light at 510 nm. Ratio measurements were performed in isotonic KCl medium. Bar indicates the presence of histamine (10 μM). The data shown are representative of 3 independent sets of experiments.

not significantly different from the 2.2 ± 0.2-fold increase in histamine-exposed cells ($P = 0.50$). From Fig. 4*A* and 4*B* it is seen that serum starvation of the cells for two hours reduces not only the basic taurine release from HeLa cells in isotonic KCl medium but also impairs the effect of histamine and ionomycin on the taurine-releasing system. It is estimated that serum starvation reduces the rate constant for the taurine efflux in isotonic KCl medium to 90 ± 4% ($n = 19$, $P < 0.001$) of the non-starved control cells, which is not significantly different from the values for the taurine efflux obtained in serum-starved cells exposed to histamine ($P = 0.50$, $n = 6$) or ionomycin ($P = 0.70$, $n = 7$).

Potentially, the impaired effect of histamine and ionomycin on the taurine-release pathway in serum-starved cells could be caused by a malfunction of the intracellular Ca^{2+}-signalling apparatus. However, from Fig. 4*C* it is seen that histamine is still able to mobilize Ca^{2+} in serum-starved cells. These findings are taken to indicate that an increase in $[Ca^{2+}]_i$ can occur in HeLa cells without any concomitant activation of the volume-sensitive taurine-efflux pathway. Thus, the effect of Ca^{2+} is not the consequence of "direct" interaction between Ca^{2+} and the taurine-releasing system, but must involve increased activity of Ca^{2+}-sensitive proteins in signaling cascades.

Involvement of CaM-regulated Proteins in the Ca^{2+} Agonist-induced Potentiation of the Volume-sensitive Taurine Leak Pathway

It has previously been demonstrated by Kirk and Kirk (1994) that the inhibition by CaM antagonists on the swelling-induced taurine efflux in HeLa cells correlates with their inhibition of CaM. To explore the role of CaM in the signaling events involved in the Ca^{2+} agonist-induced potentiation of the activity of the swelling-induced taurine efflux, we performed taurine efflux experiments in the absence and presence of the CaM antagonist W-7. In accordance with Kirk and Kirk (1994), we find that the maximal rate constant for the swelling-induced taurine efflux in hypotonic KCl medium is significantly reduced to

33 ± 2% of the control value ($n = 23$, $P < 0.001$) in the presence of 50 μM W-7 (*data not shown*). From Fig. 5*A* it is seen that treating the HeLa cells with W-7 (50 μM) for 20 min in isotonic medium and performing the subsequent efflux experiments in the absence of W-7 also reduces the maximal rate constant for the swelling-induced taurine release to 60% of the control ($n = 4$, $P = 0.02$). Furthermore, the augmentation of the swelling-induced taurine efflux following pre-exposure to the Ca^{2+} agonists histamine (10 μM) or bradykinin (1 μM) is impaired when W-7 is present during the pre-exposure period (Fig. 5*A*), i.e., the maximal rate constant for the swelling-induced taurine efflux following exposure to W-7 plus histamine ($n = 3$) or W-7 plus bradykinin ($n = 4$) is estimated at 0.19 ± 0.03 min^{-1}, which is not significantly different from the W-7-treated cells ($P = 0.2$–0.3). Numerous studies have indicated that W-7 interferes with agonist-induced Ca^{2+}-mobilization, either by causing an increase in $[Ca^{2+}]_i$ in itself (Helmeste et al., 1995; Watanabe et al., 1999; Jan et al., 2000) or by decreasing the agonist-induced Ca^{2+}-mobilization (He, Wu & Baum, 1990; Tornquist, 1993; Watanabe et al., 1999). From Fig. 5*B* it is seen that (i) W-7 (50 μM) does not in itself induce Ca^{2+}-mobilization in HeLa cells and (ii) W-7 does not inhibit the Ca^{2+}-signaling following addition of the Ca^{2+}-agonists histamine and bradykinin. It is noted that $[Ca^{2+}]_i$ following addition of the Ca^{2+} agonists stabilizes at a higher resting level in the presence of W-7, presumably due to inhibition by W-7 of the activity of CaM-regulated Ca^{2+}-ATPases in the plasma membrane (Sedova & Blatter, 1999). These data are taken to indicate that the inhibitory effect of W-7 on the Ca^{2+} agonist-induced potentiation of the activity of the taurine leak pathway reflects involvement of a CaM-regulated protein and not an impairment of Ca^{2+} agonist-induced Ca^{2+} mobilization. To identify the CaM-regulated elements we tested the effect of the CaM-dependent kinase II inhibitor KN-62, the myosin light chain kinase inhibitor ML-7 and the calcineurin inhibitor cyclosporin A on the Ca^{2+} agonist-mediated augmentation of the taurine release pathway. From Fig. 5*A* it is seen that the maximal rate constant for the swelling-induced taurine efflux is not affected by pre-exposure to KN-62 (5/10 μM) for 20 min ($n = 5$, $P = 0.13$). Moreover, the maximal rate constant for the swelling-induced taurine efflux in cells pre-exposed to either histamine ($n = 3$) or bradykinin ($n = 3$) is not significantly affected by the presence of KN-62 in the preincubation period ($P = 0.93$–0.99). Furthermore, no inhibition after application of ML-7 (10 μM; $n = 2$) or cyclosporin A (1 μM; $n = 4$) was observed on either the basic swelling-induced taurine efflux (*data not shown*) or the histamine-induced (10 μM) augmentation of the swelling-induced taurine efflux, i.e., the relative increase in the maximal rate constant for the swelling-induced taurine efflux following histamine-preexposure, is estimated at 1.20 ± 0.04 ($n = 2$) and 1.21 ± 0.08 ($n = 2$) in the

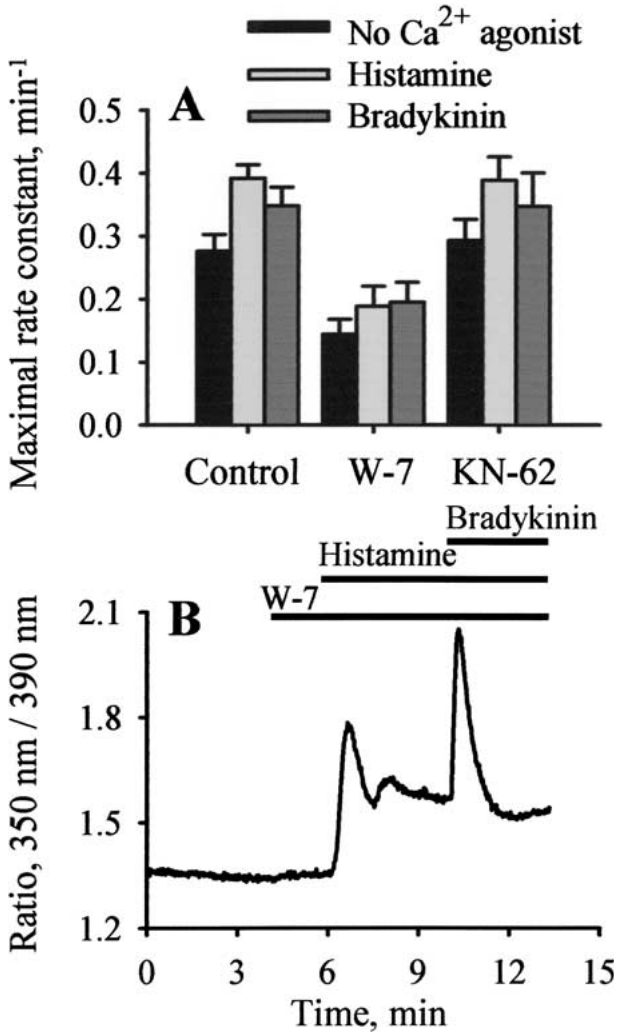

Fig. 5. The potentiating effect of pre-exposure to Ca^{2+}-mobilizing agonists on the swelling-induced taurine efflux is up-stream to calmodulin. (*A*) Cells grown to 80% confluence were preloaded with [^{14}C]-taurine for 2 h and prepared for efflux experiments as described in Materials and Methods. Before initiation of efflux experiments, cells were exposed for 10 min to either isotonic NaCl medium in the absence (*control*) or presence of W-7 (50 μM) or KN-62 (10 μM) (essentially as shown in Fig. 3*A*) prior to a further, concomitant 10 min exposure to histamine (10 μM) or bradykinin (1 μM). Efflux experiments were subsequently performed in isotonic and hypotonic NaCl medium in the absence of compounds. Rate constants for the swelling-induced taurine efflux were estimated as described in Materials and Methods, and the maximal efflux rate constants are given as mean values ± SEM based on 3–8 independent experiments. (*B*) Cells were loaded with fura-2-AM as described in Materials and Methods. The data show the ratio of the fluorescence intensities following excitation at 350 nm and 390 nm, respectively, and collection of the emission light at 510 nm. Ratio measurements were performed in isotonic KCl medium. Bars indicate the presence of W-7 (50 μM), histamine (10 μM) and bradykinin (10 μM). The data shown are representative of 3 independent sets of experiments.

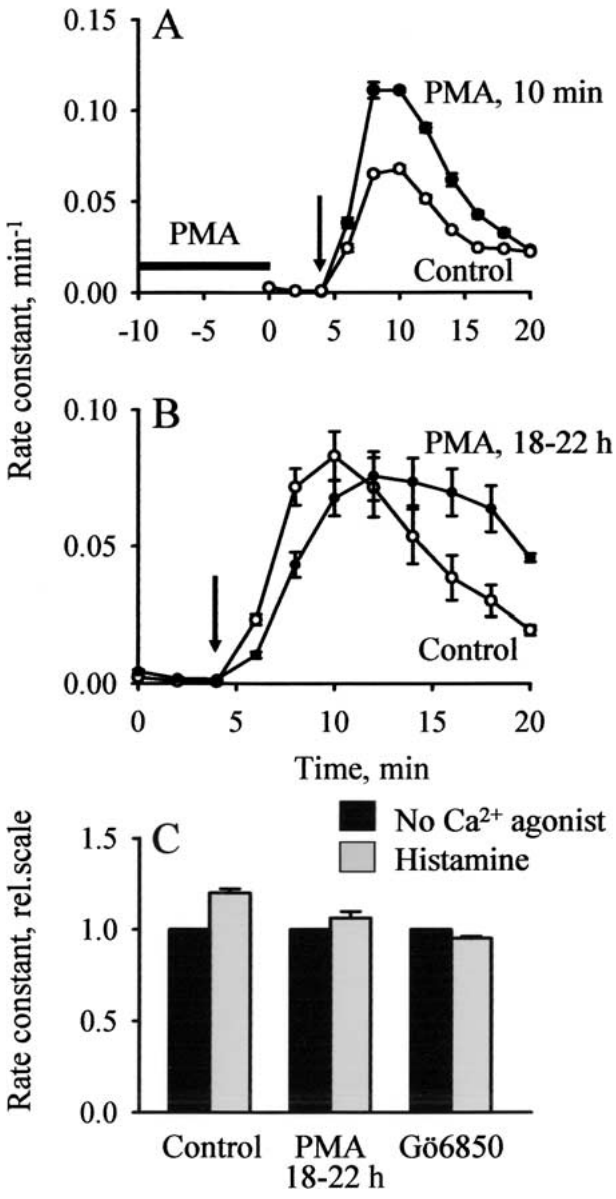

Fig. 6. PKC mediates the potentiating effect of pre-exposure to Ca^{2+}-mobilizing agonists on the swelling-induced taurine efflux. (*A–C*) Cells were grown to 80% confluence, preloaded with [^{14}C]-taurine for 2 h and prepared for efflux experiments as described in Materials and Methods. Taurine efflux measurements were performed in NaCl medium in the absence of compounds with a shift from isotonicity to hypotonicity at the time point indicated by the arrow. Rate constants were estimated as described in Materials and Methods. (*A*) Prior to initiation of efflux experiments, cells were exposed for 10 min to either isotonic NaCl medium (*empty circles*) or PMA (100 nM; *filled circles*) as indicated by the bar. The data shown are mean rate constants ± SEM based on 2 sets of independent experiments, each estimated in duplicate. Results are representative of 6 observations from 4 independent experiments. (*B*) PMA (50 nM; *filled circles*) was added directly to the growth medium 18–22 h prior to initiation of taurine efflux experiments. Data are shown as mean rate constants ± SEM based on 7 independent experiments. (*C*) Black bars indicate that cells prior to initiation of efflux experiments were exposed to either isotonic NaCl medium (*Control*; 10 min), growth medium containing PMA (50 nM; 18–22 h) or Gö6850 (2.5 μM; 1 h), whereas grey bars indicate a further 10 min exposure to histamine (10 μM), essentially as shown in Fig. 3*A*. Rate constants were obtained and the maximal efflux rate constant for the histamine-treated cells was related to the appropriate control. Data are given as mean relative values ± SEM based on 8 (control ± histamine), 4 (PMA 18–22 h ± histamine) and 4 (Gö6850 ± histamine) paired, independent experiments.

absence and presence of ML-7 and 1.22 ± 0.04 (n = 5) and 1.16 ± 0.05 (n = 4) in the absence and presence of cyclosporin A. These findings are taken to indicate that CaM-dependent kinase II, myosin light chain kinase and calcineurin are neither involved in the basic swelling-induced nor the Ca^{2+} agonist-mediated augmentation of the taurine-release from HeLa cells.

Involvement of Phorbol Ester-sensitive PKC-Isoforms in the Ca^{2+} Agonist-induced Potentiation of the Volume-sensitive Taurine Leak Pathway

Addition of histamine to HeLa cells has been shown by others to lead to activation of PKC (Smit et al., 1992; Leurs et al., 1994), and we initiated an investigation of a possible role of protein kinase C in the Ca^{2+} agonist-induced potentiation of the taurine-releasing pathway. Figure 6*A* shows that pre-incubation with phorbol 12-myristate 13-acetate (PMA, 100 nM, 10 min)—an often applied compound used to activate conventional PKC (cPKC) and novel PKC (nPKC) isoforms—potentiates the activity of the swelling-induced taurine efflux pathway in the subsequent hypotonic challenge. In accordance with the recent findings by Olivero and Stutzin (2004) we estimated from 6 observations in 4 independent sets of experiments that the maximal rate constant for the swelling-induced taurine release from HeLa cells is significantly increased 1.64 ± 0.04-fold (P < 0.05) by PMA-preexposure (100 nM, 10 min), revealing that activation of PKC mimics the effect of the Ca^{2+} agonist-induced augmentation of the swelling-induced taurine efflux. Long-term exposure to PMA has previously been reported to downregulate the activity of phorbol ester-sensitive PKC isoforms (Mellor & Parker, 1998) and from Fig. 6*B* it is seen that HeLa cells, treated with PMA (50 nM) for 18–22 h, still activate volume-sensitive taurine leak pathways following hypotonic exposure. In 7 sets of experiments it is estimated that the maximal rate constant for the swelling-induced taurine efflux in long-term PMA-treated cells is not significantly different from the control cells (P = 0.37). It is noted that a plot of the estimated rate constants for the taurine efflux versus time (Fig. 6*B*) seems to indicate a somewhat delayed inactivation of the swelling-

induced taurine leak pathway in the long-term PMA-treated cells. Whether this delay is a consequence of an impaired RVD was not further investigated. Figure 6*C* shows that the potentiation of the swelling-induced taurine efflux following histamine-pre-exposure is inhibited in long-term PMA-treated cells, i.e., following histamine pre-exposure, the relative taurine efflux from hypotonically swollen long-term PMA-treated HeLa cells is estimated at 1.06 ± 0.04 (n = 4), which is not significantly different from the relative taurine efflux from hypotonically swollen long-term PMA-treated cells (P = 0.37). These results imply a role of phorbol ester-sensitive PKC isoforms in the Ca^{2+} agonist-induced potentiation of the taurine-release pathway in HeLa cells. As both conventional and novel isoforms of PKC are activated in the presence of PMA we examined the effect of Gö6850, which is a specific inhibitor of both cPKC's and nPKC's, on the Ca^{2+} agonist-induced augmentation of the activity of the taurine-release pathway. Figure 6*C* shows that, following histamine pre-exposure, the potentiation of the swelling-induced taurine efflux is abolished by presence of Gö6850 (2.5 μM) administrated to the cells one hour prior to and during the histamine preincubation period. From 4 sets of experiments it is estimated that the maximal rate constant for the swelling-induced taurine efflux following histamine preexposure, given relative to non-histamine-treated cells, is 1.16 ± 0.02 and 0.95 ± 0.01 in the absence and presence of Gö6850, respectively. Olivero and Stutzin (2004) have recently demonstrated that Gö6976, which is a selective inhibitor of cPKC (IC_{50} < 10 nM), at 10 μM reduces the maximal rate constant for the swelling-induced taurine-release from HeLa cells by 25%. We, on the other hand, find that exposure to a lower concentration of Gö6976 (1 μM, 1 h pretreatment) has no effect on either the basic or the Ca^{2+}-agonist-induced potentiation of the swelling-induced taurine-release from HeLa cells (n = 3, *data not shown*). Thus, it is suggested that the Ca^{2+} agonist-induced potentiation of the swelling-induced taurine leak pathway in HeLa cells is mediated by an nPKC isoform.

Discussion

The mechanisms involved in the swelling-induced activation of the volume-regulating channels are diverse and seem to be highly cell type-dependent, e.g., in Ehrlich ascites tumor cells, evidence has been provided that hypotonicity initiates an intracellular signaling cascade consisting of sequential activation of phospholipase A_2 (PLA_2), release of arachidonic acid from the nuclear membrane, and oxidation of arachidonic acid by 5-lipoxygenase (5-LO) to leukotriene D_4, leading to activation of volume-sensitive K^+ channels (Hoffmann & Hougaard, 2001) and release of taurine (Lambert & Hoffmann, 1993). Similarly, it has been shown that pharmacological inhibitors of PLA_2 and 5-LO inhibit the taurine release from hypotonic-swollen HeLa cells (Lambert & Sepulveda, 2000) and NIH3T3 cells (Lambert, 2003). Reactive oxygen radicals (ROS) are produced by NIH3T3 cells (Lambert, 2003) and by pig skeletal muscle cells (Ørtenblad et al., 2003) upon hypotonic exposure, and it has been proposed that ROS, directly or indirectly via an inhibition of protein tyrosine phosphatase, prolongs the open time for the swelling-induced taurine efflux pathway (Lambert, 2004).

The present work was initiated in order to elucidate the controversial role of Ca^{2+} and Ca^{2+}-regulated proteins in the signaling cascade, which is activated by hypotonic cell swelling and results in activation of the volume-sensitive taurine leak pathway. Previous studies in various cell systems have indicated that an increase in $[Ca^{2+}]_i$ occurs upon cell swelling, although it has been questioned whether the $[Ca^{2+}]_i$ actually increases or whether it is an artifact, i.e., is secondary to a change in the affinity of Ca^{2+}-sensitive fluorophore fura-2 towards Ca^{2+} due to an altered intracellular ion strength. Using fura-2 as an indicator of global changes in $[Ca^{2+}]_i$ in HeLa cells following hypotonic exposure, we observed an initial increase followed by a stabilization at a higher resting level in the ratio of emission light (510 nm) after excitation at 350 nm and 390 nm and we originally interpreted this as a swelling-induced increase in $[Ca^{2+}]_i$. However, Grynkiewicz and co-workers (Grynkiewicz, Poenie & Tsien, 1985) have estimated the dissociation constant, k_d, for the fura-2-Ca^{2+}-complex in vitro at 224 nM in a solution containing 115 mM KCl, 20 mM NaCl, 10 mM K-MOPS (pH = 7.05, 37°C) and at 135 nM in a solution containing 100 mM KCl (pH = 7.1–7.2, 20°C). The proportion of the ion strength between the two applied in vitro solutions is similar to the reduction in intracellular ion strength one would expect when HeLa cells are transferred from isotonic (300 mOsmole/l) to a hypotonic (200 mOsmole/l) experimental medium. Correcting our obtained fura-2-ratio data for this putative reduction in k_d indicates that no change in $[Ca^{2+}]_i$ occurs in HeLa cells upon cell swelling. We suggest that the change in fura-2-emitted fluorescence upon cell swelling is due to a shift in k_d in the fura-2-Ca^{2+} complex caused by a decrease in ion strength upon dilution of cell content during cell swelling and hence, does not reflect a swelling-induced increase in $[Ca^{2+}]_i$. Similarly, Sardini and co-workers (Sardini, Sepulveda & McNaughton, 1995) could not demonstrate a change in $[Ca^{2+}]_i$ in single HeLa cells following a reduction in the extracellular osmolarity to 67% of the isotonic value. It is emphasized that in some cell lines a lack of visible global

changes in $[Ca^{2+}]_i$ does not exclude a role of Ca^{2+} in the regulation of the activity of elements in the volume-sensitive signaling cascade leading to effectuation of the RVD response. In the case of Ehrlich ascites tumor cells it has been demonstrated that induction of Ca^{2+} mobilization in association with a hypotonic exposure results in a significant acceleration of the RVD response (Hoffmann, Simonsen & Lambert, 1984; Hoffmann, 1999) even though no detectable change in $[Ca^{2+}]_i$ can be observed when Ehrlich cells are exposed to a hypotonic solution (Jørgensen et al., 1997). In the present investigation we demonstrate that the RVD response in HeLa cells is likewise accelerated in the presence of Ca^{2+} agonists (Fig. 2). As the rate of the RVD-response in many cells is limited by the K^+ permeability of the plasma membrane (Hoffmann et al., 1984; Pasantes-Morales et al., 1994; Pasantes-Morales et al., 1997), the accelerated RVD most probably reflects activation of Ca^{2+}-activated K^+ channels contributing to a faster net loss of cellular KCl and cell water. Moreover, the addition of Ca^{2+} agonists results in two independent effects on the activity of the taurine leak pathway, i.e., an augmentation of the swelling-induced taurine-release (Fig. 1*B* and Fig. 1*C*) and a faster inactivation subsequent to obtainment of maximal activity (Fig. 1*B*). This accelerated inactivation is absent in situations where the contribution of the Ca^{2+}-activated K^+ channels to the KCl loss during RVD is impaired by clamping of the outward transmembrane K^+ gradient (Fig. 1*C*) or removal of extracellular Ca^{2+} (Fig. 1*D*). As the activity of the volume-sensitive taurine leak pathway follows the changes in cell volume (Hall et al., 1996), it is assumed that the accelerated inactivation of the taurine-release pathway in the presence of Ca^{2+} agonists is secondary to an accelerated RVD due to stimulation of Ca^{2+}-activated K^+-channels and not a Ca^{2+}-mediated inhibition of the taurine leak pathway. This is further supported by the fact that addition of Ca^{2+} agonists to HeLa cells submerged in isotonic medium also increases the taurine-release (Fig. 4*B*). This is consistent with an observation made by Lambert (1998), which demonstrates that addition of the Ca^{2+}-mobilizing agonists ATP or UTP in the micromolar range increases the taurine loss from Ehrlich cells suspended in isotonic solution.

On the other hand, the increased activity of the Ca^{2+}-activated K^+-channels does not explain the augmented swelling-induced taurine release after addition of a Ca^{2+} agonist because the Ca^{2+}-activated K^+-channels are insensitive to DIDS (Hogg, Wang & Large, 1994) and dependent on extracellular Ca^{2+} (Sauve et al., 1990, 1991), whereas the Ca^{2+} agonist-induced augmentation of the swelling-induced taurine release is sensitive to DIDS (*see* Results) and independent of extracellular Ca^{2+} (Fig. 1*D*). It is therefore assumed that the increased taurine release from swollen HeLa cells in the presence of Ca^{2+} agonists (Figs. 1 and 3) and the reduced taurine release following depletion of intracellular, thapsigargin-sensitive Ca^{2+} stores (Olivero & Stutzin, 2004) reflects a shift in activity of the volume-sensitive taurine leak pathway.

Both the volume-sensitive Cl^- channel and the swelling-induced taurine release exhibit voltage dependency, i.e., the activity is increased by hyperpolarization and decreased by depolarization of the plasma membrane, and it has previously been argued that the Ca^{2+} agonist-induced increased loss of osmolytes during RVD could be secondary to a Ca^{2+}-induced hyperpolarization of the membrane due to activity of the Ca^{2+}-activated K^+ channels (Lambert, 1998). However, this can be ruled out, as the presence of Ca^{2+} agonists in either isotonic (Fig. 4*A*) or hypotonic (Fig. 1*C*) high-K^+ medium also increases the taurine loss from HeLa cells, i.e., under conditions where the membrane potential is clamped at near zero volt and net K^+ movement across the cell membrane is prevented.

These findings are taken to indicate that an increase in $[Ca^{2+}]_i$ is able to augment the activity of the volume-sensitive taurine leak pathway. In order to distinguish between a 'direct' interaction between Ca^{2+} and the taurine leak pathway or an 'indirect' Ca^{2+}-sensitive protein-mediated regulation of the taurine leak pathway, we applied two approaches, i.e., a Ca^{2+} mobilization prior to the hypotonic exposure of the cells and Ca^{2+} mobilization in serum-starved cells under isotonic condition. As demonstrated in Fig. 3*A* and *B*, a transient increase in $[Ca^{2+}]_i$ in HeLa cells prior to the hypotonic exposure 'primes' elements in the swelling-activated signaling cascade, i.e., even though $[Ca^{2+}]_i$ has returned to non-stimulated values, the hypotonic-induced taurine-release is still augmented to a similar extent as observed when the Ca^{2+} agonist was added concomitantly with the hypotonic medium (Fig. 1*B*). Furthermore, as the Ca^{2+} agonist-induced augmentation of the taurine release during isotonic conditions is completely absent in HeLa cells that have been serum-starved for two hours (Fig. 4*B*) in spite of visible Ca^{2+} mobilization (Fig. 4*C*), this suggests that the Ca^{2+} agonist-induced Ca^{2+} mobilization can be temporally isolated from an increased activity of the taurine leak pathway and that a Ca^{2+} mobilization can occur in cells without any concomitant increase in taurine release. This is taken to indicate that the stimulating effect of Ca^{2+} on the volume-sensitive taurine release pathway reflects the involvement of Ca^{2+}-regulated proteins. It is noted that the transient increase in $[Ca^{2+}]_i$ following addition of a Ca^{2+}-agonist to serum-starved cells under isotonic conditions most probably reflects release of Ca^{2+} from intracellular stores due to the reduced driving force for Ca^{2+}-influx in cells suspended in KCl medium,

where the membrane potential is clamped at near zero values.

Several Ca^{2+}-regulated proteins have been assigned a role in regulation of the volume-sensitive osmolyte channels in RVD-performing mammalian cells, e.g., $cPLA_2$, 5-LO, CaM, MLCK, IP_3-kinase and PKC. In the case of HeLa cells, pharmacological studies have indicated that activation of the volume-sensitive taurine leak pathway involves activity of a yet unidentified PLA_2, 5-LO and CaM (Kirk & Kirk, 1994; Lambert & Sepulveda, 2000), whereas electrophysiological studies have excluded a role of PKC on $I_{Cl,vol}$ (Hardy et al., 1995). Our studies confirm a permissive role of CaM in the regulation of the taurine release pathway following hyposmotic cell swelling (Fig. 5*A*). The translocation of $cPLA_2$ to the intracellular membranes and subsequent activation upon hormonal-induced stimulation requires the presence of extracellular Ca^{2+} (Hirabayashi et al., 1999) and in some cases, phosphorylation by CaMKII (Muthalif et al., 1996; Handlogten et al., 2001). However, we find that neither the basic swelling-induced nor the Ca^{2+}-potentiated swelling-induced taurine release are affected by removal of extracellular Ca^{2+} (Fig. 1*D* and Table 1) or by the presence of CaMKII-inhibitor KN-62 (Fig. 5*A*). Hence, we suggest that (i) CaMKII is not the CaM-regulated protein in the Ca^{2+} agonist-induced augmentation of the activity of the taurine leak pathway and (ii) the putative PLA_2-activity in hypotonically swollen HeLa cells does not reflect activity of $cPLA_2$. It is noted that a calcium-independent PLA_2 ($iPLA_2$) has recently been assigned a role in regulation of the swelling-induced taurine-release from NIH3T3 fibroblasts (Lambert, 2003). However, $iPLA_2$ contains a CaM-binding domain, which in its CaM-bound configuration inhibits the activity of iPLA2 (Jenkins et al., 2001). As addition of the CaM-antagonist W-7 results in inhibition, i.e., not a stimulation, of the swelling-induced taurine efflux in hypotonically swollen cells and prevents the Ca^{2+} agonist-induced potentiation of the taurine leak pathway, we suggest that $iPLA_2$ is not the CaM-regulated protein in the Ca^{2+}-activated cascade that augments the activity of the taurine-release pathway.

5-LO is regulated by Ca^{2+} via the Ca^{2+}-dependence of the C2-like structure in binding of the enzyme to the nuclear membrane (Kulkarni et al., 2002), i.e., an increase in cellular [Ca^{2+}] could lead to an increased availability of activated 5-LOs. However, as the rate-limiting step in the production of eicosanoids is in the PLA_2-mediated release of arachidonic acid, i.e., in the mobilization of the substrate for the 5-LO, we do not assume that the Ca^{2+}-sensitive target in the Ca^{2+} agonist-induced potentiation of the swelling-induced taurine-release is 5-LO.

Likewise, our data also exclude a role of MLCK and calcineurin in mediating the Ca^{2+} agonist-induced potentiation of the taurine-release pathway, as evidenced by lack of effect of ML-7 and cyclosporin A, respectively (*see* Results). It is further noted that a role of another well-known CaM-regulated protein, phosphodiesterase can also be excluded, as cAMP and PKA to our knowledge are generally not considered to participate in RVD. The latter is further supported by the fact that HeLa cells do not express endogenous cAMP-activated Cl^- current (Anderson et al., 1996). It is therefore our current opinion that the CaM-sensitive step is tightly coupled to the volume-sensitive taurine leak pathway, in analogy with the Ca^{2+}-activated K^+-channels (Saimi & Kung, 2002).

To date, the PKC family consists of ten isoforms, the conventional (cPKC) PKC_α, $PKC_{\beta I}$, $PKC_{\beta II}$ and $PKC\gamma$, the novel (nPKC) PKC_δ, PKC_ε, PKC_η and PKC_θ plus the murine PKC_μ (analogue to the human protein kinase D, PKD) and the atypical (aPKC) PKC_τ and PKC_ξ plus the murine analogue of PKC_ι, PKC_λ. They are distinguished by the activation by Ca^{2+} and diacyl glycerol (DAG) / phorbol ester (cPKC), by DAG alone (nPKC) and insensitivity towards Ca^{2+} or DAG (aPKC) (Mellor & Parker, 1998). A role of PKC has been demonstrated in the regulation of ion channel activity as well as the regulation of taurine release pathway during RVD in some cell types including HeLa cells (Chou et al., 1998; Light et al., 1998; Du & Sorota, 2000; Estevez et al., 1999; Olivero & Stutzin, 2004), but not in all (Leaney et al., 1997; Deleuze et al., 2000; Morales-Mulia et al., 2001). Figure 6*A* shows that PKC activation by addition of PMA prior to the hypotonic exposure augments the swelling-induced taurine release from HeLa cells during a subsequent hypotonic exposure, i.e., activation of PKC mimics the effects on the activity of the taurine leak pathway after addition of Ca^{2+} agonists (Fig. 3*A*). The involvement of PKC in mediation of the Ca^{2+} agonist-induced potentiation of the taurine leak pathway is further supported by the data presented in Fig. 6*C*, where it is seen that the effect of histamine is impaired following downregulation of PMA-sensitive PKC-isoforms or by the presence of the cPKC- and nPKC-inhibitor Gö6850. Thus, even though a permissive role of phorbol ester-sensitive PKC isoforms in the signaling cascade that activates the taurine leak pathway upon cell swelling cannot be detected (Fig. 6*B*), it is suggested that the Ca^{2+} agonist-induced augmentation of the taurine release from hypotonically swollen cells is mediated by phorbol ester-sensitive PKC isoforms. Addition of PMA also activates PKD (Mellor & Parker, 1998), hence the effect of PMA addition could reflect PKD activation. However, PKD is not downregulated by long-term PMA-treatment (Van Lint et al., 2002), which excludes a role of PKD in mediation of the Ca^{2+} agonist- and PMA-induced potentiation of the swelling-induced taurine release, as the Ca^{2+} agonist-in-

duced increase in taurine efflux was absent in long-term PMA-treated cells (Fig. 6*C*). Both the conventional and novel PKC isoforms (cPKC and nPKC) are submitted to regulation by PMA, however, the PKC isoform involved seems not to be of the cPKC subtype, as Gö6976, a selective inhibitor of cPKC, has no effect on either the basic or the Ca^{2+}-potentiated swelling-induced taurine efflux from HeLa cells. Hence, we suggest that a PKC isoform belonging to the novel subtype of PKC isoforms is mediating the Ca^{2+}-induced potentiation of the taurine release and that nPKC is presumably activated by the Ca^{2+} agonist-induced activation of phospholipase C_β that follows agonist binding to the receptor and that leads to DAG release.

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