Separate taurine and chloride efflux pathways activated during regulatory volume decrease

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Stutzin, Andrés, Rubén Torres, Macarena Oporto, Patricio Pacheco, Ana Luisa Eguiguren, L. Pablo Cid, and Francisco V. Sepúlveda. Separate taurine and chloride efflux pathways activated during regulatory volume decrease. Am. J. Physiol. 277 (Cell Physiol. 46): C392–C402, 1999.—Organic osmolyte and halide permeability pathways activated in epithelial HeLa cells by cell swelling were studied by radiotracer efflux techniques and single-cell volume measurements. The replacement of extracellular Cl\textsuperscript{−} by anions that are more permeant through the volume-activated Cl\textsuperscript{−} channel, as indicated by electrophysiological measurements, significantly decreased taurine efflux. In the presence of less-permeant anions, an increase in taurine efflux was observed. Simultaneous measurement of the \textsuperscript{125}I, used as a tracer for Cl\textsuperscript{−}, and \textsuperscript{3}H]taurine efflux showed that the time courses for the two effluxes differed. In Cl\textsuperscript{−}-rich medium the increase in I\textsuperscript{−} efflux was transient, whereas that for taurine was sustained. Osmosensitive Cl\textsuperscript{−} conductance, assessed by measuring changes in cell volume, increased rapidly after hypotonic shock. The influx of taurine was able to counteract Cl\textsuperscript{−} conductance-dependent cell shrinkage but only \textasciitilde 4 min after triggering cell swelling. This taurine-induced effect was blocked by DIDS. Differences in anion sensitivity, the time course of activation, and sensitivity to DIDS suggest that the main cell swelling-activated permeability pathways for taurine and Cl\textsuperscript{−} are separate.

Cell volume regulation; taurine efflux; iodide efflux; organic osmolyte

The mechanisms of cell volume regulation involve the release or uptake of intracellular organic osmolytes and ions by activation of channels and transporters. In response to swelling induced by extracellular hypotonicity, cells lose K\textsuperscript{+}, organic osmolytes, and water to recover their normal volume in a process known as regulatory volume decrease (RVD) (8, 28).

Taurine (2-aminoethanesulfonic acid) is the main organic osmolyte in many mammalian cells and is actively accumulated in various tissues at concentrations up to 65 mM (25, 34). Taurine has been shown to be released from swollen cells together with K\textsuperscript{+} and Cl\textsuperscript{−}, suggesting that it may play an important role in the RVD process (11). The molecular identity of the membrane permeability pathway used by taurine to leave the cells is not known. The pathway mediating swelling-dependent efflux of taurine appears to be shared by a variety of structurally unrelated organic molecules, catalyzes influx and efflux with similar efficiencies, does not appear to saturate with increasing concentrations of transported substance, does not present any trans effects, and is not stereoselective (11, 13). The characteristics of the transport pathway(s) are unlike those of typical membrane transporters and are more akin to those of porins.

Data suggesting that the volume-activated release of taurine is mediated by the same anion-selective channels responsible for swelling-activated Cl\textsuperscript{−} efflux have been extensively reviewed (11, 24, 28). The strongest evidence favoring a common pathway for organic osmolytes and Cl\textsuperscript{−} relies on the similar pharmacological profiles of the two effluxes and on measurements of single-channel and macroscopic currents mediated by taurine at an alkaline intracellular pH in Madin-Darby canine kidney cells (3) and in C\textsubscript{6} glioma cells (10).

Although the evidence for a common osmosensitive permeability pathway for Cl\textsuperscript{−} and organic osmolyte efflux is strong, the question of the identity of the pathways involved is not solved. Lambert and Hoffmann (15) reported the differential activation and blockade by some unsaturated fatty acids of swelling-activated taurine and Cl\textsuperscript{−} efflux in Ehrlich mouse ascites tumor cells. In addition, DIDS is a strong inhibitor of the taurine pathway in these cells but only inhibits the osmosensitive Cl\textsuperscript{−} permeability slightly. These observations suggest the presence of two distinct types of channels or permeability pathways of different natures in Ehrlich cells. For rat mammary tissue, Shennan et al. (27) also demonstrated that taurine efflux activated by cell swelling seems to occur via a pathway different from the dominating volume-sensitive anion channel. Sánchez-Olea et al. (26) noticed that in three different cell lines there was a poor correlation between the abilities of a variety of blockers to inhibit swelling-induced organic osmolyte and \textsuperscript{125}I efflux, an observation inconsistent with the single common pathway hypothesis. Emma et al. (7) have considered, to explain RVD dependence on intracellular electrolytes, that Cl\textsuperscript{−} channels separate from a taurine-permeable pathway could provide the main route for swelling-activated Cl\textsuperscript{−} efflux. Recently, we have shown that replacing extracellular Cl\textsuperscript{−} with poorly permeant anions differentially modulates the volume-activated Cl\textsuperscript{−} current and taurine efflux in HeLa cells (29). As for the Ehrlich cells, we have also observed that DIDS is a stronger inhibitor of taurine efflux than of Cl\textsuperscript{−} conductance. These observations are inconsistent with a single common pathway for osmosensitive taurine and Cl\textsuperscript{−} efflux.
In this report we extend our previous observations on the dependence of swelling-activated taurine permeability on extracellular anions. In addition, we study the time course of activation of taurine permeability after cell swelling and compare it with that of the osmosensitive Cl– permeability. Our data suggest that the main taurine and Cl– permeability pathways activated during RVD are separate.

**MATERIALS AND METHODS**

Cells. HeLa cells were grown at 37°C in a 5%-95% CO2-air atmosphere in DMEM (with no taurine added) supplemented with 5% FCS, 80,000 IU/l penicillin, and 50 µg/ml streptomycin.

Efflux experiments. Cells were grown to 80% confluence (usually 48–72 h after plating) in 35-mm-diameter, six-well plates. They were loaded with [3H]taurine (1 µCi/ml) alone or with 125I (0.5 µCi/ml) by incubation for 2–2.5 h at 37°C in culture medium without FCS. Before each efflux experiment, the loading solution was removed and the cells washed three times at room temperature with isosmotic medium (see the figure legends for the compositions of the solutions). The medium contained in each well (0.75 ml) was replaced at timed intervals and saved for counting. Cells were allowed to equilibrate for 3–5 min and then were challenged with hypotonic solutions. The radioactivity remaining in the cells at the end of the experiment was determined after disruption of the cells with 0.75 ml of 0.25 M NaOH. Samples were counted in beta and gamma scintillation counters, and appropriate corrections were applied to obtain pure 3H radioactivity. In isotonicity, the amounts of [3H]taurine and 125I remaining in the cells decreased following single-exponential decay functions. The data were expressed as efflux rate constants calculated from the equation

\[ k_i = \frac{cpm_i}{\left(\sum_{j=1}^{n} cpm_j \right) / 2 / \tau} \]

where \( k_i \) is the efflux rate constant at time \( i \) and \( cpm_i \) is the radioactivity counts per minute at time \( i \), in an experiment where \( n \) samples are taken; \( \tau \) is the time interval for sample \( i \) in minutes. All the experiments were carried out at room temperature (24°C).

Electrophysiology. For electrophysiological experiments, cells were seeded onto 35-mm-diameter cell culture petri dishes, which could be directly mounted on the stage of an inverted microscope. Standard whole cell patch-clamp recordings were performed as described before (5), except that the amplifier was used in the current-clamp mode.

Cell volume measurements. Changes in cell water volume in single cells were assessed by measuring changes in the concentration of an intracellularly trapped fluorescent dye (1). HeLa cells grown as for efflux experiments were plated on round coverslips, loaded with calcine-AM (5 µM for 5 min), and then superfused with isosmotic solution for 30 min before the experiment was started. The experiments were performed with a confocal laser imaging system (Carl Zeiss). Excitation light was 488 nm, and emitted light was measured at wavelengths longer than 515 nm. Pictures were obtained at 10-s intervals, and the fluorescence of a ~10-µm² area in the center of a cell was measured. The records were corrected for fluorescence decay independent of cell volume changes (primarily due to dye photobleaching). The data are presented as \( V/V_0 \), where \( V_0 \) = cell water volume in isosmotic solution at time 0 and \( V \) = cell water volume at time \( t \). This was calculated from \( F/F_0 \) (\( F \) = fluorescence intensity) exactly as described previously (1).

Reagents. [3H]taurine was obtained from American Radio-labeled Chemicals (St. Louis, MO), and 125I was obtained from the Comisión Chilena de Energía Nuclear. All other reagents were of analytical grade and were purchased from Sigma (St. Louis, MO) and Merck (Darmstadt, Germany).

**RESULTS**

Cell swelling-activated taurine efflux depends on extracellular anions. The cell swelling-activated Cl– conductances of HeLa and other cells have been demonstrated to increase in the presence of anions more permeant than Cl– in the extracellular medium. The converse is observed for impermeant anions, such that the predicted flux through the channel should be directly correlated with the permeability of the main anion present in the medium (29, 30, 33). To address the question of the influence of different extracellular anions on the volume-activated taurine efflux, tracer experiments were conducted. Cells loaded with [3H]taurine were used to monitor amino acid loss (29). After a period to measure basal efflux rate, the medium bathing the cells was exchanged for a 30% hypotonic solution to assess the increase in taurine permeability brought about by cell swelling. Hypotonic solutions in which NaCl was replaced by KCl (or the corresponding anion) and gramicidin were used to clamp the membrane potential at ~0 mV and thus preclude the possible effects of anion replacement on membrane potential. As shown in Fig. 1, top, there was a marked increase in the rate constant for taurine efflux in Cl–-containing medium. The permeability reached an apparently steady level in 5 min. When Cl– was replaced by the impermeant anions F– or glutamate, there was an increase in the values of permeability reached. On the other hand, when permeant anions were used, there was a decrease in the maximal efflux rate achieved (Fig. 1, bottom). Anion replacement could directly affect the membrane transporter/channel mediating taurine efflux or could affect the mechanisms signaling the permeability change. In an attempt to distinguish these possibilities, the speeds of increase in the rate constant in the presence of different anions (I– and glutamate, i.e., those producing maximal decrease in the speed of increase) were normalized to compare them with that in the presence of Cl–. Figure 1, top, inset, shows that the anion composition of the medium did not alter significantly the speed of increase of the efflux rate constant, suggesting that the mechanism transducing the change in volume signal into permeability activation was not affected.1 Swelling-activated taurine permeability is therefore dependent on the extracellular anion, following the sequence (derived from \( k_{10 \text{ min}} \) values) glutamate > F– > Cl– > NO3– > Br– > SCN– > I–. This sequence contrasts with the permeability sequence SCN– > I– > Br– > Cl– > F– > glutamate, described for HeLa and a variety of other

1 Exposure of cells to a high K+ concentration probably prevents RVD, but a secondary swelling might be expected in the presence of permeant anions. This would lead to an underestimation of the inhibitory effect of the permeant anions described here.
might account for the observed differences. A method widely used to infer cell swelling-induced increases in anion conductance is to monitor the efflux of $^{125}$I used as a surrogate for Cl$^-$. The rates of taurine and iodide efflux in the same cells were therefore monitored by double labeling with $^3$H]-taurine and $^{125}$I, and the effects of cell swelling and anion replacement were studied.

Volume-activated taurine and I$^-$ effluxes follow different time courses. If taurine and Cl$^-$ share a common pathway, it would be expected that volume-activated taurine and iodide effluxes should follow similar time courses and be similarly affected by anion replacement. Under control, isotonic conditions HeLa cells lost previously loaded $^{125}$I and $^3$H]-taurine with time courses well described by a single-exponential decay function (29). In isotonicity, the respective rate constants were 0.05–0.08 min$^{-1}$ for $^{125}$I and 0.01–0.02 min$^{-1}$ for $^3$H]-taurine (Fig. 2; note the use of separate y-axes). The rate constants remained unaltered in the isosmotic medium for up to 14 min and were not affected by the replacement of extracellular Cl$^-$ by glutamate. However, challenging the cells with hypotonic solution markedly increased the rates of efflux for both compounds. Figure 2A shows an experiment in which a high (105 mM) Cl$^-$ concentration was maintained throughout. The increase in efflux rate was transient for iodide but appeared sustained for the amino acid within the time frame of the experiments. When the hypotonic challenge experiment was performed in a low-Cl$^-$, glutamate-rich hypotonic solution (Fig. 2B), an approximately similar increase in the efflux rate constant for iodide was observed. The increase in taurine efflux, however, was three-fold larger than that in the Cl$^-$-rich hypotonic solution. For both compounds the increase was transient, the fall in iodide efflux being faster in glutamate medium but not in Cl$^-$ medium. The decrease in permeability for I$^-$ could be adjusted to single-exponential functions with constants of 0.27 ± 0.04 min$^{-1}$ (Cl$^-$-medium) and 0.74 ± 0.09 min$^{-1}$ (glutamate medium). The increase in taurine permeability was transient in glutamate medium but not in Cl$^-$ medium. This might be due to RVD taking place in low-Cl$^-$ glutamate medium because of the larger outwardly directed Cl$^-$ gradient. In both hypotonic conditions, $^{125}$I efflux reached its maximal value before $^3$H]-taurine efflux, a result also shown by others (19). One possible explanation for the more transient activation of $^{125}$I efflux would be that the cells become depleted of this tracer more rapidly than they do of $^3$H]-taurine. However, this was not the case: for experiments in Cl$^-$ medium, radioactivity levels remaining in the cell at the end of the experiments were 20 ± 1% and 16 ± 1% for $^3$H]-taurine and $^{125}$I, respectively. Corresponding figures in glutamate medium were 6 ± 0.5% and 15 ± 1%.

Even in the event that taurine and I$^-$ were sharing a common pathway, changes in membrane potential during the experiment would be expected to affect I$^-$ but not taurine efflux, if this amino acid were leaving the cell in the neutral form. This could account for the differing time courses for the efflux rates after hypo-

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**Fig. 1. Swelling-activated taurine efflux depends on extracellular anion composition of the medium.** Shown are time courses of taurine efflux rate constants in HeLa cells and effect of hypotonicity with different Cl$^-$ replacements. After 4 min in isotonic NaCl-rich extracellular solution (composition in mM): 70 mannitol, 105 NaCl, 5 KCl, 0.5 MgCl$_2$, 1.3 CaCl$_2$, and 10 Tris-HEPES (pH 7.4, 316 mosmol/l), taurine-loaded cells were exposed to hypotonic (198–205 mosmol/l) solutions containing KCl or other potassium salts as indicated, in the absence of any other monovalent cations. All hypotonic solutions contained 10 µM gramicidin to fix membrane potential to the K$^+$ equilibrium potential ($E_k$). Data are mean efflux rate constants ($n = 3$) ± SE. In experiment with glutamate medium, that with highest efflux rate, radioactivity remaining in cells at end of experiment was 22 ± 2%. Inset: comparison of normalized rates of increase of efflux in Cl$^-$, I$^-$, and glutamate (symbols stand for same anions as in main graphs). These were calculated by subtracting basal efflux rate and normalizing for efflux at 10 min, time at which maximal efflux was reached: normalized $\Delta k = (k_t - k_{min})/k_{10\ min}$ where k is rate constant at time indicated by subscript. Arrows indicate change from isotonic to hypotonic medium.

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Cell types for the volume-activated Cl$^-$ current (5, 17, 18).

The observed contrast between the anion sequences in their effects on the volume-activated Cl$^-$ current and taurine efflux suggests that the effects might occur via different transmembrane pathways. The conditions under which ion currents are measured differ significantly from those used to assess taurine efflux, and this
tonic stimulus. To explore this possibility, experiments were conducted in the presence of high K⁺ concentrations (replacing Na⁺) and the cation ionophore gramicidin. Under these conditions the potential should be determined by the K⁺ gradient and should therefore be effectively damped around 0 mV throughout. As can be seen in Fig. 3, the differences in time course for the development of I⁻ and taurine effluxes after hypotonic stimulus were still present under these conditions. Activation of I⁻ efflux was transient, being maximal at the fourth minute after stimulus. Maximal activation of taurine efflux was not reached within the time frame of the experiment. In these experiments the radioactivity remaining in the cells at the end of the experiment was 24 ± 1% for [³H]taurine and 24 ± 2% for ¹²⁵I. The observation that I⁻ and taurine effluxes follow different
time courses, in addition to the differential effects of external Cl\textsuperscript{−} on their efflux rates, suggests that there must be different permeability pathways involved.

Although I\textsuperscript{−} has been extensively used as surrogate for Cl\textsuperscript{−} in efflux experiments, the equivalence in behavior of both halides has not been proven. I\textsuperscript{−} has been shown to affect the onset of activation of Cl\textsuperscript{−} current by cell swelling (30) and to have anomalous effects on cystic fibrosis transmembrane conductance regulator, another Cl\textsuperscript{−} channel (31). In addition, the different accessibility of I\textsuperscript{−} to Cl\textsuperscript{−} channels, perhaps a consequence of intracellular binding, might also complicate its use as tracer for Cl\textsuperscript{−}. One way to avoid these potential problems is to infer Cl\textsuperscript{−} conductance from cell volume decrease. However, this experimental design requires Cl\textsuperscript{−} permeability to be rate limiting for volume change, a condition that can be met by exposing the cells to gramicidin in the absence of external permeant cations. This maneuver will hyperpolarize the cells, and it has been suggested that the volume-activated taurine permeability is voltage dependent (14). The effect of hyperpolarization on the magnitude of the taurine efflux rate and on the time course of activation in HeLa cells was therefore tested.

Cell swelling-activated taurine efflux is voltage dependent. The voltage dependence of taurine efflux was studied by swelling the cells with a NaCl-containing hypotonic solution. Once taurine efflux was activated, the cells were exposed to hypotonic solutions containing different K\textsuperscript{+} concentrations [all Na\textsuperscript{+} replaced by N-methyl-D-glucamine (NMDG)] and gramicidin. Figure 4, top, shows an experiment in which taurine efflux was activated by hypotonicity in the usual manner. After 5 min in hypotonic medium, the cells were subjected to hypotonic solutions containing different concentrations of K\textsuperscript{+} to give final K\textsuperscript{+} concentrations indicated. Bottom: efflux rate constant for taurine measured at 10 min is plotted as a function of membrane potential measured in separate whole cell current-clamp experiments. Means ± SE of 6 experiments are shown. Remaining levels of [\textsuperscript{3}H]taurine radioactivity at end of experiments were 27 ± 2% (105 mM K\textsuperscript{+}), 13 ± 1% (25 mM K\textsuperscript{+}), 16 ± 1% (5 mM K\textsuperscript{+}), and 12 ± 1% (0 mM K\textsuperscript{+}).

Fig. 3. Hypotonically induced \textsuperscript{125}I and [\textsuperscript{3}H]taurine efflux in high-K\textsuperscript{+} medium containing gramicidin. HeLa cells were preloaded with \textsuperscript{125}I and [\textsuperscript{3}H]taurine, and then efflux was measured as a function of time. Solution bathing cells was switched from standard experimental solution (composition in legend to Fig. 2) to a hypotonic solution (standard experimental solution but omitting mannitol, replacing all NaCl by KCl, and adding 10 \textmu M gramicidin; 204 mosmol/l) at time indicated by arrow. Results are means ± SE (shown when falling outside symbol) of 3 experiments. Note separate axes for \textsuperscript{125}I and [\textsuperscript{3}H]taurine efflux data.

Fig. 4. Effect of membrane potential on taurine efflux. Taurine-loaded cells were exposed to isotonic (iso) and hypotonic conditions as indicated at top. Hypotonic challenge was then applied in standard NaCl solution (see legend to Fig. 1). After 5 min, with a steady rate of taurine efflux having been established, solution was switched to similarly hypotonic solutions in which all NaCl (and KCl) was replaced by mixtures of KCl and N-methyl-D-glucamine (NMDG) chloride (with 10 \textmu M gramicidin) (hypo gramicidin 0 Na\textsuperscript{+}) to give final K\textsuperscript{+} concentrations indicated. Bottom: efflux rate constant for taurine efflux measured at 10 min is plotted as a function of membrane potential measured in separate whole cell current-clamp experiments. Means ± SE of 6 experiments are shown. Remaining levels of [\textsuperscript{3}H]taurine radioactivity at end of experiments were 27 ± 2% (105 mM K\textsuperscript{+}), 13 ± 1% (25 mM K\textsuperscript{+}), 16 ± 1% (5 mM K\textsuperscript{+}), and 12 ± 1% (0 mM K\textsuperscript{+}).
cantly higher at hyperpolarized potentials. The fact that taurine efflux is dependent on membrane potential might indicate that a charged form of the amino acid is transported. It has been argued, however, that taurine must be leaving cells mainly in the neutral form, judging from the fact that a small change or no change in intracellular pH occurs concomitantly (15). Alternatively, an intrinsic voltage dependence of the transporter or channel involved could account for our observations. In view of the effect of $V_m$ on the rate constant of taurine efflux, the question of whether the activation process of volume-induced taurine efflux was also affected by hyperpolarization was addressed. Cells were swollen in a control (NaCl) hyposmotic solution or in a solution of NMDG chloride plus gramicidin. Figure 5 shows that, as expected, the efflux rate constant reached was higher in gramicidin-NMDG chloride solution than in NaCl-rich solution, consistent with the expected depolarization and hyperpolarization, respectively. However, by normalizing the NaCl to the experiment with gramicidin-NMDG chloride, a similar time course for the development of permeability was seen.

These results demonstrate that although swelling-induced taurine permeability is voltage dependent, the time course of its activation is unaffected by hyperpolarization. This makes it possible to compare the time courses of activation of swelling-dependent $\text{Cl}^-$ conductance, studied under hyperpolarized conditions, with that for the taurine efflux pathway.

Cell swelling activates separate $\text{Cl}^-$- and taurine-prefering permeability pathways. To compare the time courses of development of taurine permeability and $\text{Cl}^-$ conductance, experiments were conducted to monitor cellular volume by a single-cell, fluorescence-based method (1). The approach uses the intracellularly trapped fluorescent dye calcein. Figure 6, top, shows a record of $F_0/F_t$ in normal (NaCl) isotonic medium and the effect of brief ($\sim 2$ min) exposures to anisosmotic solutions. Fast changes of fluorescence intensity were observed for all anisosmotic challenges, reflecting concentration or dilution of the dye within the element of volume under consideration. $F_0/F_t$ values are plotted against the osmotic pressure change ($\pi_0/\pi_t$) in Fig. 6, bottom. The linear relationship between these two variables gave a slope of 0.48 [correlation coefficient ($r$) = 0.99], indicating that 52% of the dye was trapped in an intracellular compartment insensitive to changes in osmolality. This was used to correct data to yield changes in cell water volume ($V_t/V_0$) after correction for bound dye (see text). Line predicts behavior of a perfect osmometer.

Fig. 5. Effect of hyperpolarization on time course of activation of taurine efflux. HeLa cells were preloaded with taurine, and then efflux was measured as a function of time. Cells were originally bathed in isotonic solution and then switched to a hypotonic one (arrow). Solutions used were standard NaCl hyposmotic solution (○; see legend to Fig. 1 for composition) or a hypotonic solution of the same tonicity but with all cations replaced by NMDG and containing 10 μM gramicidin (□). Data for NaCl hyposmotic solution (○) are shown also after normalization to efflux rate value seen at 7 min in NMDG chloride-gramicidin. Results are means ± SE (shown when falling outside symbol) of 3 experiments.

Fig. 6. Osmotic behavior of HeLa cells in anisosmotic media. Cells were loaded with calcein, and fluorescence ($F_t$; subscripts indicate time) of single HeLa cells was monitored. Top: experiment in which cells were bathed initially in standard isotonic solution (see legend to Fig. 1 for composition) that was switched, for brief intervals marked by boxes, to solutions of increased or decreased osmotic pressure (obtained by addition or removal of D-mannitol). Bottom: reciprocals of steady-state changes in fluorescence, $F_0/F_t$, derived from top are shown plotted against respective osmotic pressure change ($\pi_0/\pi_t$). Data are also plotted as changes in cell water volume ($V_t/V_0$) after correction for bound dye (see text). Line predicts behavior of a perfect osmometer.
which corresponds to dye trapped in an osmotically insensitive compartment, compares with 0.66 for C6 glioma cells and 0.7 for NG108-15 neuroblastoma × glioma hybrid cells (1). Figure 6, bottom, also shows that calculated V/V0 data compared well with an ideal line predicting the behavior of a perfect osmometer.

Figure 7 shows experiments to estimate swelling-activated Cl− permeability by monitoring cell volume. The rationale of the experiment is to make Cl− conductance rate limiting for net ion transport across the plasma membrane. This is achieved by the replacement of all monovalent cations in the medium by the impermeant cation NMDG and the addition of gramicidin. Under these conditions the conductance of the membrane is dominated by the K+ permeability and, should a Cl− conductance be present, the cell will shrink at a rate proportional to anion permeability. Figure 7, top, shows that under isotonic conditions cell volume remained constant. When the switch to an isotonic gramicidin-NMDG chloride solution was made, little change in volume was detected. This indicates that under isosmotic conditions the permeability for Cl− is low. However, on exposure to a 30% hypotonic solution, the cells immediately swelled, indicating that cell volume was being adequately monitored in these experiments.

The time course for the activation of the swelling-induced Cl− conductance was studied as shown in Fig. 7, bottom. Cell swelling was triggered with a 30% hypotonic NaCl solution. Under these conditions cells underwent a slow, partial RVD. The slow rate of RVD is probably due to the fact that the experiment was conducted at room temperature, at which swelling-activated K+ conductance develops slowly (23). In addition, cultured HeLa cells have an intracellular taurine concentration of only ~14 mM (21), discarding a major contribution of this amino acid to the RVD process. At different times (arrows), the hypotonic NaCl solution was exchanged for a hypotonic gramicidin-NMDG chloride solution. At all times, a fast decrease in cell volume occurred, indicating that Cl− conductance was present from 1–10 min after hypotonic shock. The time course of cell volume decrease, which gives an estimate of Cl− conductance, could be fitted to monoexponential decay functions. The derived rate constants indicate that as early as 1 min after cell swelling the Cl− conductance had already reached full activation and started to decrease.

These results are in contrast with the time course observed for taurine efflux. Whereas in the 1- to 4-min time frame taurine permeability increased from 0 to 0.42 min⁻¹, Cl− conductance, measured as the rate of decrease in volume, fell from 0.60 to 0.48 min⁻¹. The simplest interpretation of this result is that a separate taurine permeability activates with a delay compared with the Cl− conductance. If this conclusion is correct, adding extracellular taurine to swollen cells should not alter the rapid decreases in cell volume seen in Fig. 7 within the first 3–4 min after hypotonic exposure. At later times, however, taurine permeability should activate, and the decrease in cell volume would be predicted to be counteracted by amino acid entry into the cells. Experiments shown in Fig. 8 address this point. Cell volume was monitored as described before, and, after swelling in 30% hypotonic NaCl medium occurred, the solution was switched at the times indicated (arrows) to 30% hypotonic gramicidin-NMDG chloride solution containing either 120 mM taurine or sucrose, a disaccharide not expected to permeate through the taurine permeability pathway. Cells challenged with gramicidin-NMDG chloride at 1 min into hypotonicity shrunk rapidly regardless of whether taurine or sucrose was present in the external solution (Fig. 8, A and B). However, in those cells exposed to 120 mM external taurine, volume decrease was arrested 3–4 min after hypotonicity and a secondary swelling often started to occur ~7 min after hypotonicity (Fig.
This suggests that under the conditions of the experiment, the taurine permeability pathway activated 3–4 min after cell swelling. In agreement with this, cells challenged with gramicidin-NMDG chloride at 5 min (Fig. 8C) or 10 min (Fig. 8D) after hypotonic shock showed little decrease in volume in taurine-containing medium compared with cells in sucrose-containing medium.
taurine replacing sucrose, shrinkage was arrested 4–5 min after hypotonic stimulation and a secondary swelling took place. The same protocol was carried out with a solution in which 50 µM DIDS had been added to the taurine-rich solution. With the stilbene in the medium, shrinkage in the presence of taurine occurred in a manner indistinguishable from that in sucrose solution, suggesting that the swelling-activated taurine pathway had been completely blocked by DIDS without any measurable effect on the main swelling-activated Cl\(^{-}\) conductance.

**DISCUSSION**

In the present work we have used HeLa epithelial carcinoma cells to study the external anion dependence and time course of activation of cell swelling-dependent permeability for taurine and contrasted them with the same characteristics for osmosensitive Cl\(^{-}\) conductance. HeLa cells have been demonstrated to undergo RVD when swollen by exposure to hypotonic solutions (32). Electrophysiological recordings have shown that they possess Cl\(^{-}\) and K\(^{+}\) channels activated by cell swelling and intracellular Ca\(^{2+}\), respectively, which have been postulated to mediate KCl loss leading to volume recovery (4, 5). HeLa cells swollen hypotonically increase their taurine permeability and also their permeability to iodide. There is a remarkable similarity in pharmacological sensitivity between these two effluxes (12) and also an apparent good correlation with the pharmacology of swelling-activated anion channels (5). Swelling-activated anion channels of HeLa cells correspond to what have been termed VSOAC (volumesensitive organic osmolyte anion channels) (28), which are postulated to be responsible also for swelling-induced intracellular organic osmolyte release during RVD. Recent work, however, points to differences in pharmacological sensitivity and other characteristics between the two processes (7, 15, 26, 27) that might suggest heterogeneity. Our previous work also suggested that HeLa cells could have more than one volume-activated permeability pathway for taurine and Cl\(^{-}\) on the basis of their differential modulation by external Cl\(^{-}\) (29, 30). Now we have extended this to a range of anions and in addition present evidence that the main Cl\(^{-}\) conductance and taurine permeability pathways must be separate on the basis of their different time courses of activation after hypotonic shock.

We have postulated that Cl\(^{-}\) regulates the swelling-activated anion channel by interaction with a site of unknown location within it so that its activity depends on the presence of the anion (29, 30). This type of gating has been demonstrated by elegant work on the ClC-0 Cl\(^{-}\) channel expressed in amphibian oocytes (22). The opening of the channel is strongly favored by the presence of extracellular Cl\(^{-}\); other less permeant anions are less effective in supporting channel activity, and the reverse is true of more permeant anions. Pusch et al. (22) propose a novel mechanism, in which gating is intrinsically linked to permeation, to account for the data. Possibly a similar mechanism accounts for the

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2 In addition, we have explored the effect of DIDS on swelling-activated \(^{125}\)I efflux and found a \(K_i\) value of 20 µM (in high extracellular Cl\(^{-}\) solution; result not shown), a fivefold difference in sensitivity compared with swelling-activated taurine efflux. This is a higher sensitivity than expected if Cl\(^{-}\) were permeating through the cell swelling-activated conductance and might suggest that this halide is not an adequate surrogate for Cl\(^{-}\). Clarification of this point will have to await further experiments.
anion dependence of the activity of swelling-activated anion channels. If the same conductance mediates organic osmolyte efflux, it would be expected to affect similarly taurine permeability. The cell swelling-activated taurine efflux, however, shows an almost inverse effect when a series of anion replacements is used. This suggests that the pathway mediating the efflux of amino acid is different from the swelling-activated, outwardly rectifying Cl⁻ channel. In addition, it suggests that the osmosensitive organic osmolyte permeability pathway can be modulated by extracellular inorganic anions.³

Much of the evidence put forward in support of the contention that the osmosensitive taurine efflux pathway is identical to the swelling-activated outwardly rectifying Cl⁻ channels in HelA and other cells comes from the comparison of organic osmolyte and iodide efflux. In the present work ³²P efflux was measured concomitantly with taurine movement. I⁻ efflux was little affected by extracellular Cl⁻ removal, whereas in the same cells taurine efflux was increased considerably. Other marked differences in the behavior of the two effluxes suggest heterogeneity of the pathways mediating the effluxes of these compounds. Iodide efflux reaches a maximum at 2 min after hypotonic challenge, whereas taurine efflux is maximal only after 4–6 min. The effect of hypotonicity on permeability was, however, much more transient for I⁻ efflux than for taurine efflux. Taurine efflux was still elevated at 6–10 min after hypotonicity, remaining elevated within the time frame of the experiment. For I⁻, on the other hand, the efflux rate had returned essentially to basal levels during the same time. When Cl⁻ was replaced by glutamate, the effect of hypotonicity on efflux was transient for both compounds but the differences in rate of development of the full effect remained: the I⁻ peak efflux rate was reached within 2–3 min, whereas it took 4–5 min for taurine efflux to peak. The reestablishment of control values after the maximum effect was also faster for I⁻. These differences are difficult to explain if the halide and the amino acid are leaving the cell through a single pathway.

It might be reasonably argued that the conditions under which patch-clamp whole cell recordings are carried out are so different from those for intact cell efflux measurements as to render comparison unwarranted. Also, I⁻ behaves differently from Cl⁻, as observed in electrophysiological studies (30, 31). For these reasons the time course of activation of Cl⁻ conductance was studied by measuring Cl⁻ conductance-limited changes in cell volume (9). These experiments demonstrated that swelling-induced Cl⁻ conductance is fast to develop, reaching full activation within 1 min of hypotonic shock.⁴ The counteraction of RVD by extracellular amino acids can be used to assess their permeability (20). Using this approach, we provide further evidence for the difference in time courses of activation of Cl⁻ conductance and taurine permeability after hypotonic shock. Whereas the main Cl⁻ conductance activates rapidly (<1 min) after hypotonic cell swelling, the taurine permeability pathway activates with a delay of 3–4 min. We have previously shown that the stilbene DIDS is a reasonably good inhibitor of the swelling-activated taurine permeability with K₁₉ values of 0.2 and 4 µM depending on the extracellular Cl⁻ concentration (29). On the other hand, DIDS is a voltage-dependent inhibitor of the volume-activated Cl⁻ channel, being particularly weak at hyperpolarizing potentials (2, 5). In Ehrlich cells DIDS has been shown to block swelling-induced taurine but not Cl⁻ flux (15). The results reported above demonstrate that DIDS abolished the taurine permeability without affecting the main swelling-activated Cl⁻ conductance measured simultaneously in the same cell under experimental conditions favoring hyperpolarization. These data, taken together, are better interpreted by assuming that separate permeability pathways mediate the main Cl⁻ and taurine osmosensitive fluxes. DIDS was able to inhibit I⁻ efflux, albeit at higher concentrations than those for inhibiting taurine permeability. This is in contrast with the apparent insensitivity of Cl⁻ conductance to the stilbene and might indicate that I⁻ is a poor surrogate for Cl⁻ in cell swelling-sensitive pathways.

The experimental evidence and the conclusion reached in the present work appear at variance with the great deal of evidence suggesting that cell swelling-activated Cl⁻ channels (often referred to as VSOAC) are the organic osmolyte channels mediating the osmosensitive efflux of taurine and other intracellular substances. The excellent reviews that recently appeared on the subject also point out the existence of other Cl⁻ channels that might participate in the RVD process and that do not correspond to the so-called VSOAC (11, 13, 28). Recent work has identified the cloned Cl⁻ channel CIC-3 as the putative molecular counterpart of VSOAC, but there is no information as to its taurine permeability (6). It is therefore possible that, for taurine and other organic osmolytes, osmosensitive efflux is via an anion channel but that a separate channel accounts for the main pathway for Cl⁻ efflux under hyposmotic conditions. The sensitivity of taurine permeability to membrane potential observed here and reported by others previously (15) would be perhaps better ex-

³ An alternative explanation for this result, as already proposed previously (29), is that anions interact with taurine within a single permeability pathway, which may be a multiple-occupancy anion channel, so that residency by low-permeability anions would favor taurine flux and the converse would occur with permeable anions. Interactions between acidic amino acids and Cl⁻ within the pores of volume-regulated Cl⁻ channels have recently been demonstrated (16), but remain to be explored for nonanionic amino acids or other organic osmolytes.

⁴ There is a discrepancy between the apparent time courses for activation and inactivation of a swelling-activated anion conductance estimated from cell volume experiments and those from the ³²P experiments. We ignore the reason for this discrepancy, but I⁻ is more permeant than Cl⁻ through swelling-activated Cl⁻ channels and would be expected to exit more rapidly than the native anion during hypotonicity. This will alter the ratio of the concentration of I⁻ to that of Cl⁻ during efflux, which could affect apparent permeability.
plained by a channel being the membrane agency mediating the efflux of the amino acid.

In conclusion we would like to propose, as a working hypothesis to explain our data, that cells possess at least two different permeability pathways with differing selectivities: a strictly anion-selective channel of early activation during RVD and a permeability allowing some halide permeation. The latter pathway might allow some halide permeation. Both pathways could be ion channels, but there is no reason to assume that taurine efflux is not carrier mediated.

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