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Volume Regulation in Human Fibroblasts: Role of Ca^{2+} and 5-Lipoxygenase Products in the Activation of the Cl^- Efflux

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Abstract. Trypsinized human skin fibroblasts in suspension perform regulatory volume decrease (RVD) after cell swelling in hypotonic medium. During RVD, $^{36}Cl^-$ efflux is dramatically increased and the cell membrane is depolarized, indicating the activation of Cl^- channels. This activation of Cl^- channels depends on extracellular as well as on intracellular Ca^{2+}. The swelling-induced Cl^- efflux and the RVD response are inhibited by the 5-lipoxygenase inhibitor ETH 615-139. Finally, following hypotonic treatment, cellular pH decreases. The pH decrease does not involve the Cl^-/HCO_3^- exchange because it is independent of the external Cl^- concentration.

Key words: Regulatory volume decrease — Volume regulation — Cl^- channel — 5-Lipoxygenase — Leukotriene-D_4

Introduction

It has recently been reported that the net loss of KCl observed during regulatory volume decrease (RVD) of human fibroblasts, following exposure to hypotonic medium, involves the activation of separate conductive K^+ and Cl^- pathways (Rugolo et al., 1989; Mastrocola, Flamigni & Rugolo, 1991). The Cl^- channels activated by cell swelling have a low conductance (3 pS) and they remain open for several minutes (Rothstein & Bear, 1989; Mastrocola et al., 1991). Although the transport pathways involved in RVD have been thoroughly investigated in several different kinds of cells (*see* Hoffmann & Kolb, 1991; Hoffmann & Ussing, 1992; Hoffmann, Simonsen & Lambert, 1993), the mechanisms of the activation are only partly understood. An increase in the cytoplasmic Ca^{2+} concentration, caused by release from intracellular stores and/or by entry across the plasma membrane, seems to be a major activator of KCl efflux. Calcium is part of a signal cascade proposed to be involved in RVD in Ehrlich ascites tumor cells (Hoffmann et al., 1993). This cascade includes stimulation of the inositol phosphate cycles (Christensen et al., 1988; Hoffmann et al., 1993), increased activity of protein kinase C (A.K. Larsen, B.S. Jensen and E.K. Hoffmann, *unpublished results*) and increase in leukotriene-D4 (Lambert, Hoffmann & Christensen, 1987; Lambert, 1993). In many cell types, stretch-activated ion channels have been described (*see* Morris, 1990), and it has been proposed that their function might be related to Ca^{2+} influx through the plasma membrane during RVD (Christensen, 1987; Lansman, Hallam & Rink, 1987). Opening of such nonselective stretch-activated cation channels after cell swelling has been directly demonstrated in Ehrlich cells (Christensen & Hoffmann, 1992).

In the present study, we report that trypsinized human skin fibroblasts exposed to hypotonic medium partially recover their cell volume within 8–10 min. The activation of $^{36}Cl^-$ efflux during RVD is significantly reduced by removal of extracellular calcium and by depletion or buffering of intracellular Ca^{2+}. We also suggest that the effect of Ca^{2+} ions might be related to the activity of the 5-lipoxygenase (5-LO), a Ca^{2+}-dependent enzyme which is involved in the conversion of arachidonic acid to leukotrienes. Finally, we demonstrate that intracellular pH decreases during RVD, through the action of a mechanism which does not involve the DIDS-sensitive Cl^-/HCO_3^- exchanger.

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Materials and Methods

Cell Culture

Human skin fibroblasts, derived from healthy individuals, were maintained in Dulbecco's modified Eagle's medium containing 2 mM L-glutamine, 10% fetal calf serum and antibiotics, incubated at 37°C in a CO_2 incubator with humidified atmosphere of 5% CO_2 in 95% air. Cells were subcultured weekly by trypsinization. For the experiments with cells in suspension, fibroblasts were treated for 2–4 min at 37°C with Ca^{2+}- and Mg^{2+}-free balanced salt solution containing 0.25% trypsin and 0.1% EDTA. Cells were then diluted with the growth medium, centrifuged and resuspended in the incubation medium.

Incubation Media

The standard experimental medium (300 mOsm/kg, adjusted with mannitol) had the following composition (in mM): 135 NaCl, 3 KCl, 1.3 $CaCl_2$, 1.5 $MgCl_2$, 10 D-glucose, 20 NaHEPES and pH was adjusted to 7.4. In Cl^--free gluconate medium, Cl^- was substituted by the equimolar amount of gluconate. In choline medium, Na^+ was substituted by the equimolar amount of choline. The hypotonic medium (200 mOsm/kg) was prepared from the standard experimental medium by dilution with 20 mM NaHEPES. In hypotonic medium with low Cl^- (7 mM instead of 95 mM), NaCl was substituted by the equimolar amount of Na-gluconate.

Cell Volume Measurements

For cell volume measurements, cells were trypsinized, centrifuged and resuspended in the incubation medium. Aliquots of the cell suspension were diluted 500-fold with the experimental solution (filtered with 0.45 μm filter, Millipore) to give a final cell density of about 20,000 cells/ml (i.e., cytocrit 0.008%). Cell volume distribution curves were obtained using a Coulter counter model ZB, tube orifice 140 μm, equipped with a Coulter channelyzer (C-1000). The mean cell volume was calculated as the median of a volume distribution curve. Absolute cell volumes in fl (10^{-15} liter) were calculated from the median of the cell volume distribution curves using polystyrene latex beads (diameter 13.5 μm, Coulter Electronics) as standard.

Fluorimetric Measurements

Fluorimetric measurements were performed in thermostated polystyrene cuvettes on a Perkin Elmer LS-5 luminescence spectrometer. *Membrane potentials* were estimated from the fluorescence intensity of the dye 1,1'-dipropyloxadicarbocyanine ($DiOC_3$-(5)). Cells were trypsinized, centrifuged and resuspended in the experimental solution, at a cell density equal to 600,000 cells/ml. The dye was added at a final concentration of 3.6 μM. A stable fluorescence signal was achieved within 15 min. Excitation and emission wavelengths were 577 and 605 nm, respectively, and slit widths were 5 nm. Calibration was performed with the cation ionophore gramicidin in Na^+-free/Cl^- choline media by isosmotically varying the extracellular K^+ concentration. The calibration curve was a plot of the fluorescence after addition of gramicidin *vs.* the Nernst equilibrium potentials for K^+. The depolarization induced by hypotonic dilution was estimated from the difference in fluorescence between cell groups diluted with an equal volume of "dye-free" isotonic medium (control) or 20 mM NaHEPES (hypotonic), using the slope of a standard curve in which all samples were diluted to 66% with an identical but dye-free solution (for details, *see* Lambert, Hoffmann & Jørgensen, 1989). *Intracellular pH* was assessed by use of the acetoxymethylester of a fluorescent probe (BCECF-AM). Cells (10^5) were seeded on 10 x 50 mm glass coverslips and allowed to grow to confluence (4–5 days). Cells were then washed with phosphate buffered solution (PBS), and incubated at 37°C for 30 min with the incubation medium containing BCECF-AM at a final concentration of 5 μM. Cells were washed twice with PBS-containing bovine serum albumine (BSA, 1 mg/ml), to remove excess probe, and the cells were subsequently incubated in dye-free incubation medium at 37°C. BCECF leakage from the cells at 37°C was estimated at 0.18%/min. Excitation wavelength was 495 nm, emission wavelength was 525 nm and slit widths were 5 nm. Calibration was carried out in KCl medium using the K^+/H^+ ionophore nigericin for dissipation of the pH gradient across the membrane (intracellular pH = extracellular pH) and using TRIS and TES as titrants. The calibration curve was obtained by simultaneous recordings of pH in the cuvette and the fluorescence (*see* Thomas et al., 1979; Kramhøft, Lambert & Hoffmann, 1988).

$^{36}Cl^-$ Efflux

The $^{36}Cl^-$ efflux experiments were performed as described in Mastrocola et al. (1991). Cells (1.2 x 10^5) were seeded into each 9.6 cm^2 well of a Nunc six-well culture plate and allowed to grow to confluence in 3 ml of growth medium for 5–7 days. For the experiments, cells were washed twice with 10 ml/well of 150 mM NaCl and 10 mM NaHEPES (pH 7.4) (300 mOsm/kg), and incubated with the incubation medium containing 2 μCi/ml of $Na^{36}Cl$ for 90 min, in a water bath at 37°C. Efflux was determined as follows: after 15 sec, the efflux medium was removed from a well and transferred to a scintillation vial for counting. An equal volume of the same medium was added to the well within 2–3 sec. This process of removing efflux medium and adding fresh efflux medium was repeated at the indicated time points. At the end of the experiment, the monolayer was dissolved in 1 ml of 0.2 M NaOH (15 min at 55°C). Aliquots of the cell lysate were collected for determination of radioactivity and protein content (Bradford, 1976). The total radioactivity in the cells at zero time was calculated by summing the radioactivity in each aliquot of the efflux medium plus the radioactivity remaining in the cells. The rate constant (k) for $^{36}Cl^-$ efflux was determined from the experimental points, fitted according to a one-compartment model, by a computer program based on standard nonlinear least-squares procedure (Bevington, 1969).

Reagents

$Na^{36}Cl$ was from Amersham. Stock solutions of $DiOC_3$-(5) iodide (1.2 mM)(Molecular Probes, Junction City, OR), ETH 615-139 (2 mM)(donated by Dr. I. Ahnfelt-Rønne, Leo Pharmaceuticals, Copenhagen) and NDGA (50 mM) (Sigma, St. Louis, MO) were prepared in ethanol and kept at −20°C. BCECF-AM (1 mg/ml) (Molecular Probes, Junction City, OR) and BAPTA/AM (2 mM) (Calbiochem) were prepared in dry dimethyl sulfoxide (DMSO). Leukotriene-D_4 was purchased from Cascade Biochem, Berkshire, UK. DIDS was from Sigma.

Statistical Evaluation

All values are expressed as the mean ± SEM with the number of experiments in brackets; P is the level of significance on a Student's t-test on paired observations.

Abbreviations

HEPES, N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid; TRIS,tris (hydroxymethyl) aminoethane; TES, n-tris (hydroxymethyl) methyl-2-aminoethane-sulfonic acid; $DiOC_3$-(5), 1,1'-dipropyloxadicarbocyanine; BCECF-AM, 2,7-bis(2-carboxyethyl)-5(and-6)carboxy-fluorescein, tetraacetoxymethyl ester; NDGA, nordihydroguaiaretic acid; ETH 615-139, N-substituted quinolylmethoxy phenylamine; LTD_4, 5S-hydroxy-6R- S-cysteinyl, glycinyl-7,9-trans-11,14-cis-eicosatetraenoic acid. 5-HPETE, 5-hydroperoxy eicosatetraenoic acid; DIDS, 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid; BAPTA/AM, acetomethoxy derivative of 1,2.bis (2-aminophenoxy) ethane N,N,N',N' tetraacetic acid.

Results

Regulatory Volume Decrease (RVD) in Human Fibroblasts

It has previously been shown that exposure of human fibroblasts to hypotonic medium activates a conductive Cl^- pathway (Mastrocola et al., 1991). To assess whether the opening of the channel was actually related to the RVD response, we measured the changes in cell volume of fibroblasts in suspension after exposure to hypotonic medium. Figure 1 shows that, following reduction of the medium osmolality from 300 to 200 mOsm/kg, the cells swell within the first minute as almost perfect osmometers, i.e., their volume increases 35 ± 2% (n = 11) over the isotonic cell volume. Subsequently, the cells shrink towards their original cell volume with about 60% recovery obtained after 10 min. The gluconate medium was chosen to avoid entrance of NaCl via a Na^+,Cl^- or $Na^+,K^+,2Cl^-$ cotransport system, which in this case could counteract the swelling-induced net loss of KCl and look like an inhibition of the RVD response. It is noted that although the mean cell volume in isotonic medium of the two fibroblast lines, used in the present investigation differed from each other, i.e., 5,256 ± 147 fl (n = 5) and 4,164 ± 131 fl (n = 6), the time course of the RVD response was the same in both cell lines.

Membrane Potential during RVD

It has previously been shown that in human fibroblasts the Cl^- conductance exceeds the K^+ conductance under hypotonic conditions (Mastrocola et al., 1991). This is expected to result in a depolarization of the cell membrane during RVD. The membrane-potential-sensitive fluorescent dye $DiOC_3$-(5) was used to monitor changes in the membrane potential during RVD, and the results are shown in Fig. 2. Dilution of the cell suspension with either isotonic medium or 20 mM NaHEPES initially reduces the fluorescence to 66%, followed by a redistribution of the dye, which is so fast that we are not always able to follow it. If the cell swelling is not accompanied by any change in membrane potential, the resulting external fluorescence would be slightly lower than in the suspension diluted with isotonic medium (*see* Lambert et al., 1989). However, dilution of the cell suspension with 20 mM NaHEPES is accompanied by a large increase in fluorescence, which reaches a new steady-state within 5 min (Fig. 2), indicating a depolarization of the cell membrane. The mean value of the membrane potential for human fibroblasts in isotonic medium was estimated at -60 ± 12 mV (n = 3) and the magnitude of the depolarization induced by hypotonic dilution was estimated at 34 ± 4 mV (n = 3).

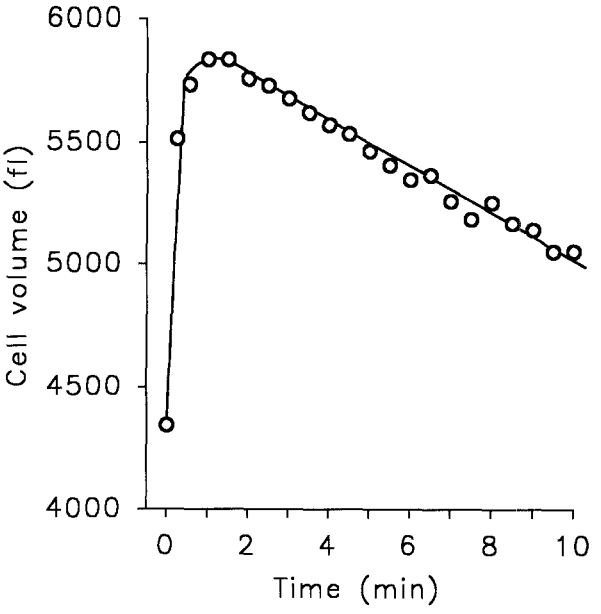

Fig. 1. Cell volume recovery in human fibroblasts exposed to hypotonic medium. At zero time, an aliquot of cells in isotonic solution (300 mOsm/kg) was transferred to hypotonic gluconate medium (200 mOsm/kg) giving a final cell density of about 20,000 cells/ml. The change in cell volume was followed with time in a Coulter counter. The figure is representative of 11 independent experiments.

Effect of Ca^{2+} on Volume-Activated $^{36}Cl^-$ Efflux

The Table illustrates the effect of Ca^{2+} removal from the hypotonic medium on the rate constant (k) of

Table. Effect of extracellular and intracellular Ca^{2+} on the rate constant of $^{36}Cl^-$ efflux from human fibroblasts

Conditions	Rate constant for swelling-induced $^{36}Cl^-$ efflux (relative to control)	
Ca^{2+}-containing	1	
Ca^{2+}-free	0.75 ± 0.09	($P < 0.05$)
Ca^{2+}-depleted	0.51 ± 0.03	($P < 0.001$)
Ca^{2+}-containing + BAPTA	0.58 ± 0.05	($P < 0.01$)

Cells were loaded with $^{36}Cl^-$ as described in Materials and Methods. The rate constants of the $^{36}Cl^-$ efflux are given relative to control ± SEM of at least three independent sets of experiments. The control $^{36}Cl^-$ efflux under *Ca^{2+}-containing* conditions was measured in hypotonic gluconate medium containing 1.3 mM $CaCl_2$. The $^{36}Cl^-$ efflux under *Ca^{2+}-free* conditions was measured in hypotonic Ca^{2+}-free efflux medium containing 0.2 mM EGTA. Under *Ca^{2+}-depleted* conditions, after $^{36}Cl^-$ loading, cells were kept for 15 min in a Ca^{2+}-free incubation medium containing $^{36}Cl^-$ and 1 mM EGTA; $^{36}Cl^-$ efflux was measured in hypotonic Ca^{2+}-free medium containing 0.2 mM EGTA. Where indicated, 10 μM BAPTA/AM was added to the incubation medium containing $^{36}Cl^-$ and kept for 40 min. *P* is the value of significance in a Student's *t*-test where the values are tested against the control value 1.

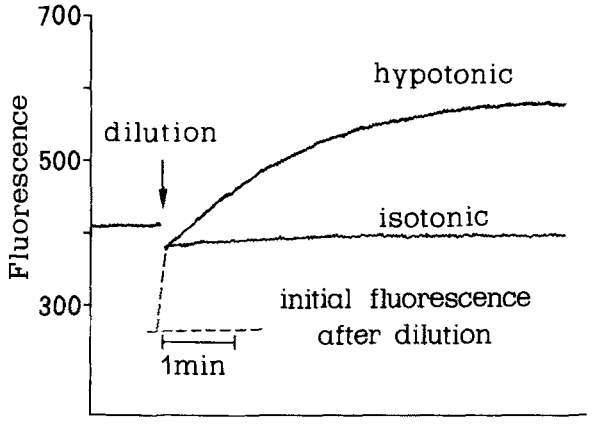

Fig. 2. $DiOC_3$-(5) fluorescence after isotonic and hypotonic dilution of human fibroblasts in NaCl medium. Human fibroblasts were equilibrated with the fluorescent dye $DiOC_3$-(5) (3.6 μM, cell density 600,000 cells/ml) for 15 min in isotonic NaCl medium after which a stable fluorescence signal was obtained. At the time indicated by the arrow, the cell suspension was diluted with dye-free isotonic medium or dye-free 20 mM NaHEPES (final osmolality: 200 mOsm/kg), and the fluorescence was recorded as a function of time. The initial fluorescence was calculated from the degree of dilution (66%). The traces represent seven identical sets of experiments.

the swelling-induced Cl^- efflux. A significant reduction of the rate constant is observed in nominally Ca^{2+}-free efflux medium containing 0.2 mM EGTA. A more substantial reduction is obtained by depleting intracellular Ca^{2+} with 15 min incubation in Ca^{2+}-free 1 mM EGTA-containing medium (Table). Under this condition, the level of intracellular free Ca^{2+} of human fibroblasts has been reported to fall to 20 nM, being the resting $[Ca^{2+}]_i$ 60 nM in standard Ca^{2+}-containing medium (Johnson et al., 1990). The Table shows that a similar reduction of $^{36}Cl^-$ efflux in hypotonic medium was obtained by treating fibroblasts with 10 μM BAPTA/AM, an intracellular Ca^{2+} buffer (Tsien, 1980).

Effect of 5-Lipoxygenase Inhibitors on $^{36}Cl^-$ Efflux and RVD

It was previously shown that hypotonic swelling of Ehrlich ascites tumor cells was associated with stimulation of leukotriene synthesis (Lambert et al., 1987). If leukotrienes are also involved in the activation of Cl^- efflux during RVD in human fibroblasts, inhibitors of 5-LO should inhibit RVD, as well as the swelling-induced $^{36}Cl^-$ efflux. As shown in Fig. 3, the 5-LO inhibitor ETH 615-139 (Kirstein, Thomsen & Ahnfelt-Rønne, 1991) completely inhibits the volume recovery after cell swelling at a concentration of 5 μM. Figure 4 shows the effect of ETH 615-139 on $^{36}Cl^-$ efflux from human fibroblasts suspended in isotonic medium and after transfer to hypotonic medium. It is seen that the efflux of $^{36}Cl^-$ is dramatically stimulated by the hypotonic treatment and that this stimulation is completely blocked by ETH 615-139. ETH 615-139 also seems to slightly reduce $^{36}Cl^-$ efflux under isotonic conditions. The concentration of ETH 615-139 required to inhibit the $^{36}Cl^-$ efflux (20 μM) is higher than the concentration required for the cell volume measurements because the amount of cells used for $^{36}Cl^-$ efflux experiments is higher than the amount used in Coulter counter experiments. Finally, the 5-LO inhibitor

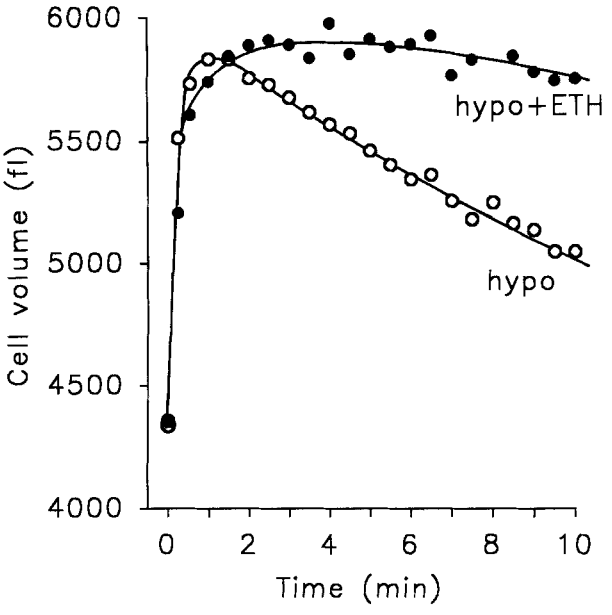

Fig. 3. Effect of the 5-lipoxygenase inhibitor ETH 615-139 on RVD in human fibroblasts. Experimental protocol as in Fig. 1. Open symbols represent control cells. Filled symbols represent cells with 5 μM ETH 615-139 added at time zero. The figure represents eight independent sets of experiments.

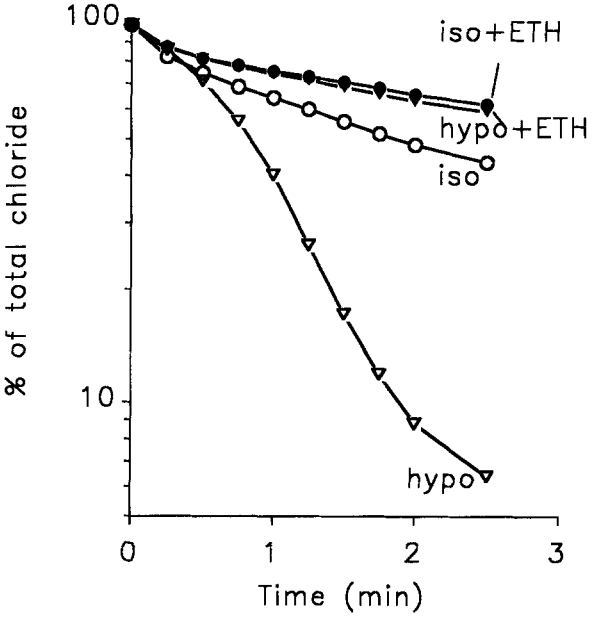

Fig. 4. Effect of the 5-lipoxygenase inhibitor ETH 615–139 on $^{36}Cl^-$ efflux from human fibroblasts. Cells treated as described in the Table were exposed to isotonic gluconate medium (○) and hypotonic gluconate medium (▽, 200 mOsm/kg). When indicated, cells were pretreated with ETH 615–139 (20 μM) for 1 min before exposure to isotonic (●) or hypotonic (▼, 200 mOsm/kg) media containing 20 μM ETH 615–139. The Cl^- content of fibroblasts (100%) was estimated at 504 ± 46 nmol/mg protein (n = 17). The curves indicate three identical sets of experiments. Percent of tracer remaining in cells was calculated for each time point and plotted on a log scale as a function of time.

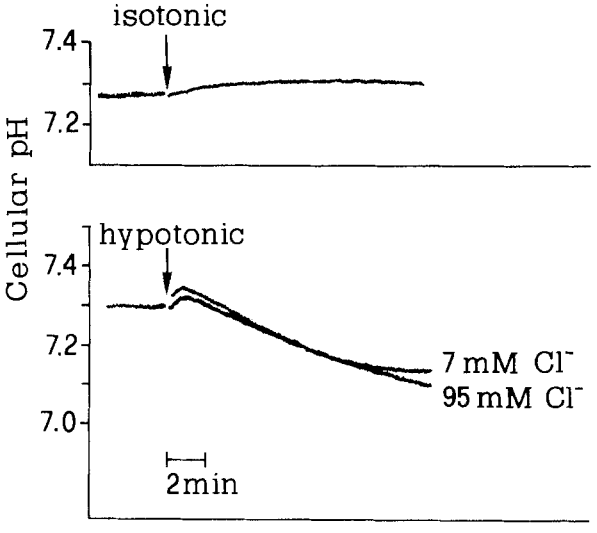

Fig. 5. Changes in intracellular pH (pH_i) in human fibroblasts after exposure to hypotonic medium. Cells grown on coverslips and loaded with BCECF-AM as described in Materials and Methods, were exposed to isotonic (300 mOsm/kg) or hypotonic (200 mOsm/kg) medium with 7 mM Cl^- or 95 mM Cl^-. The extracellular pH was 7.4. The figure is representative of eight independent experiments for normal hypotonic medium, and four independent experiments for low Cl^- hypotonic medium.

NDGA (70 μM) inhibited the rate constant for $^{36}Cl^-$ efflux to 65 ± 5% (n = 3) (*data not shown*).

Effect of Added LTD_4 on RVD and $^{36}Cl^-$ Efflux

The activation of 5-LO leads to the formation of 5-HPETE, which in turn could be converted into several types of leukotrienes: LTA_4, LTB_4, LTC_4, LTD_4 and LTE_4 (Needleman et al., 1986). In particular, LTD_4 has been shown to be responsible for Cl^- channel activation in Ehrlich ascites tumor cells (Lambert, 1987, 1989; Lambert et al., 1987). LTD_4 (100 nM) was neither able to accelerate the RVD response nor to induce shrinkage of human fibroblasts in isotonic medium (*data not shown*). LTD_4 also failed to increase the rate constant for $^{36}Cl^-$ efflux both under hypotonic and isotonic conditions (*data not shown*).

Effect of Hypotonic Shock on Intracellular pH

Previous data obtained in blood platelets (Livne, Grinstein & Rothstein, 1987) and in Ehrlich ascites tumor cells (Livne & Hoffmann, 1990) show that cells undergoing RVD acidify. The pH indicator BCECF was used to determine intracellular pH in human fibroblasts. Figure 5 shows that after expo-

sure to hypotonic medium, the intracellular pH (pH_i) is initially slightly increased, but after 1 min a gradual acidification (0.20 ± 0.016 pH unit in 10 min, n = 8) takes place. The acidification might be caused by recycling of extracellular Cl^- for cellular HCO_3^- via the anion exchanger (Livne & Hoffmann, 1990). To assess the involvement of the anion exchanger, the extracellular concentration of Cl^- in the hypotonic medium was reduced from 95 to 7 mM, which is well below the cytoplasmic concentration after cell swelling. At this Cl^- concentration, the Cl^- gradient is outwardly directed and the Cl^-/HCO_3^- exchanger is therefore unable to contribute to the acidification. Figure 5 shows that the acidification rate is not reduced under these conditions. The addition of the anion-exchanger inhibitor DIDS (250 μM) to the hypotonic medium also failed to reduce cytoplasmic acidification (*data not shown*).

Discussion

It has previously been shown that exposure of human fibroblasts to hypotonic conditions leads to activation of conductive pathways for Cl^- and K^+ (Mastrocola et al., 1991). In the present study, we have followed the changes in cell volume of trypsinized human fibroblasts using a Coulter counter. Human fibroblasts initially swell as almost perfect osmometers, but subsequently recover their volume within approximately 8–10 min (Fig. 1). This regulation is a consequence of the activation of Cl^-- and K^+-conductive pathways, resulting in net loss of KCl and osmotic obliged cell water. The progressive increase in the fluorescence of the membrane-potential-sensitive probe $DiOC_3$-(5) after hypotonic dilution (Fig. 2) clearly indicates that the cell membrane depolarizes during RVD. This finding confirms that the increase in the Cl^- conductance in human fibroblasts following hypotonic exposure exceeds the concomitant increase in the K^+ conductance (Mastrocola et al., 1991). A similar effect of hypotonic dilution on the fluorescence signal has also been demonstrated with Ehrlich ascites tumor cells (Lambert et al., 1989) and with human lymphocytes (Grinstein et al., 1982). In Ehrlich ascites tumor cells both the activation of Cl^- channels and the activation of nonselective cation channels contribute to the depolarization (Christensen & Hoffmann, 1992). Whether nonselective cation channels are involved in the swelling-induced depolarization in fibroblasts is presently unknown.

Several factors have been proposed to play a role in the activation of the RVD response in different kinds of cells including Ca^{2+}, calmodulin, eicosanoids, phospholipases, protein kinases and the microfilament network (*see* Hoffmann et al., 1993; Lambert, 1993). In several cell types, Ca^{2+} entry across the cell membrane is required for the swelling-induced RVD response. In other cell types, the volume response is unaffected by removal of external Ca^{2+}, and in these cells swelling-induced release of Ca^{2+} from internal stores has been proposed to be involved in the RVD response (Pierce & Politis, 1990; McCarty and O'Neil, 1992; Hoffmann et al., 1993). The finding that the rate constant for $^{36}Cl^-$ efflux from human fibroblasts exposed to hypotonic Ca^{2+}-free medium was reduced by 25% (Table) suggests that Ca^{2+} entry across the plasma membrane contributes to the expected increase in cellular Ca^{2+} during RVD. In Ehrlich ascites tumor cells, an intact microfilament network is a prerequisite for a normal RVD response in Ehrlich cells (Cornet, Lambert & Hoffmann, 1993), and Ca^{2+} influx seems to be mediated via nonselective stretch-activated cation channels (Christensen & Hoffmann, 1992). Release of Ca^{2+} from intracellular stores also seems to play a significant role as depletion of fibroblast Ca^{2+} results in a dramatic reduction in the rate constant for $^{36}Cl^-$ efflux (Table). In this respect, it is noteworthy that EGTA might inhibit RVD by causing nonspecific effects on the cell membrane. This possibility, however, is unlikely since we have demonstrated (Table) that the intracellular Ca^{2+} chelator BAPTA, which increases the intracellular buffering capacity, also inhibits Cl^- efflux. This latter finding suggests that an increase in $[Ca^{2+}]_i$ is required for RVD in fibroblasts. It thus seems that Ca^{2+} influx as well as Ca^{2+} release from intracellular stores are involved in the activation of conductive Cl^- transport in hypotonically swollen human fibroblasts. This is in agreement with observations from several other cellular systems (for reviews, *see* Pierce & Politis, 1990; McCarty & O'Neil, 1992; Hoffmann et al., 1993). It should be noted that a reduction in the cellular Ca^{2+} concentration might reduce the Cl^- efflux indirectly by closing the Ca^{2+}-activated K^+ channel. In this case, the membrane of the human fibroblasts will become more depolarized and the driving force for the Cl^- efflux reduced. However, this possibility can be ruled out since depletion of intracellular Ca^{2+} does not reduce the $^{36}Cl^-$ efflux from human fibroblasts under isotonic conditions (T. Mastrocola and M. Rugolo, *unpublished data*).

A direct effect of Ca^{2+} on cell-swelling-activated Cl^- channel was not observed with patch-clamp experiments in Ehrlich cells (Christensen & Hoffmann, 1992) and in bovine chromaffin cells (Doroshenko & Neher, 1992). It has therefore been proposed that the effect of Ca^{2+} might be indirect and mediated by Ca^{2+}-related metabolic processes (Hoffmann & Kolb, 1991; Doroshenko & Neher, 1992). In relation

to this, it has recently been reported that an increased synthesis of leukotrienes via the 5-LO system is a prerequisite for the RVD response in the Ehrlich cells (Lambert et al., 1987). It is well known that in resting cells the 5-LO is localized in the cytosol and, upon elevation of $[Ca^{2+}]_i$, it is translocated to the membrane and becomes activated (Rouzer et al., 1990). It is therefore possible to suggest that the effect of Ca^{2+} on RVD of fibroblasts might be related to the activation of the 5-LO. In this respect, it is noteworthy that the addition of the 5-LO inhibitor ETH 615-139 to human fibroblasts completely inhibits the activation of the $^{36}Cl^-$ efflux after hypotonic cell swelling (Fig. 4) and the concomitant regulation of the cell volume (Fig. 3). These results are consistent with the hypothesis that one or more 5-LO products are likely to be involved in the activation of the Cl^- channels in human fibroblasts. In Ehrlich cells it has been shown that it is LTD_4 which is responsible for the activation of Cl^- channels (Lambert, 1987, 1989; Lambert et al., 1989) as well as for the stimulation of the taurine leak pathway (Lambert & Hoffmann, 1993) during RVD. Indeed, addition of LTD_4 to Ehrlich cells results in a net loss of KCl and cell water, which under hypotonic conditions is seen as a dramatic acceleration of the RVD response and under isotonic conditions as a significant cell shrinkage (Lambert, 1987, 1989; Lambert et al., 1987). Conversely, in human fibroblasts LTD_4 does not accelerate the RVD response nor induce cell shrinkage under isotonic conditions (*not shown*). This suggests that Cl^- conductance in human fibroblasts during RVD and in isotonic medium is not increased by LTD_4, as confirmed by the lack of effect of LTD_4 on the rate constant of Cl^- efflux under both isotonic and hypotonic conditions. Further studies are required to identify the 5-LO products responsible for activation of the Cl^- channels.

In human fibroblasts, like in other cell types (Livne et al., 1987; Livne & Hoffmann, 1990), cytoplasmic acidification follows RVD (Fig. 5). The reduction in the extracellular Cl^- concentration of the hypotonic medium and the addition of the inhibitor of the Cl^-/HCO_3^- exchanger were without effect on the acidification, suggesting that the Cl^-/HCO_3^- exchanger is not involved. The cytoplasmic acidification might be the result of HCO_3^- extrusion through the Cl^- transport pathway activated by osmotic cell swelling, although acid production by activation of K^+/H^+ exchange, as described for *Amphiuma* red cells (Cala, 1985), should also be considered. The selectivity of the volume-sensitive anion transport pathway has not been investigated in human fibroblasts. In MDCK cells (Weiss & Lang, 1992) and in human epithelial cells (Rasola et al., 1992) it has been reported that the volume-sensitive anion transport pathway is permeable for both HCO_3^- and Cl^-.

Although the cytoplasmic acidification observed during RVD seems to be a widely occurring phenomenon, its possible role in RVD has not been defined. However, it can be postulated that a pH change might be a signal which affects the cellular metabolism (Watford, 1990).

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