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On the Role of Calcium in the Regulatory Volume Decrease (RVD) Response in Ehrlich Mouse Ascites Tumor Cells

N.K. Jørgensen[1], S. Christensen[2,3], H. Harbak[2], A.M. Brown[4], I.H. Lambert[1], E.K. Hoffmann[1], L.O. Simonsen[2]

[1]Biochemical Department, The August Krogh Institute, University of Copenhagen, Universitetsparken 13, DK-2100 Copenhagen Ø, Denmark
[2]Department of Cellular and Molecular Physiology, The August Krogh Institute, University of Copenhagen, Universitetsparken 13, DK-2100 Copenhagen Ø, Denmark
[3]Institute of Physiology, University of Aarhus, Aarhus, Denmark
[4]Department of Physiology and Pharmacology, University of Nottingham, Nottingham, U.K.

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Abstract. The putative role for Ca^{2+} entry and Ca^{2+} mobilization in the activation of the regulatory volume decrease (RVD) response has been assessed in Ehrlich cells. Following hypotonic exposure (50% osmolarity) there is: (i) no increase in cellular Ins(1,4,5)P_3 content, as measured in extracts from [2-^{3}H]*myo*inositol-labeled cells, a finding at variance with earlier reports from our group; (ii) no evidence of Ca^{2+}-signaling recorded in a suspension of fura-2-loaded cells; (iii) Ca^{2+}-signaling in only about 6% of the single, fura-2-loaded cells at 1-mM Ca^{2+} (1% only at 0.1-mM Ca^{2+} and in Ca^{2+}-free medium), as monitored by fluorescence-ratio imaging; (iv) no effect of removing external Ca^{2+} upon the volume-induced K^+ loss; (v) no significant inhibition of the RVD response in cells loaded with the Ca^{2+} chelator BAPTA when the BAPTA-loading is performed in K^+ equilibrium medium; (vi) an inhibition of the swelling-induced K^+ loss (about 50%) at 1-mM Ba^{2+}, but almost no effect of charybdotoxin (100 nM) or of clotrimazole (10 μM), reported inhibitors of the K^+ loss induced by Ca^{2+}-mobilizing agonists. Thus, Ca^{2+}signaling by Ca^{2+} release or Ca^{2+} entry appears to play no role in the activation mechanism for the RVD response in Ehrlich cells.

Key words: Volume regulation — Phosphoinositidase C — Ins(1,4,5)P_3 — Ca^{2+}signaling — K^+ channels — Charybdotoxin — Clotrimazole

Correspondence to: L.O. Simonsen

Introduction

Nearly all animal cells when swollen in hypotonic media have the capability to recover their volume by regulatory volume decrease (RVD), involving a net loss of cellular osmolytes and cell water. In most cell types the major part of the net solute loss during the RVD response is accounted for by KCl loss mediated by activation of separate K^+ and Cl^- channels, with an additional loss of organic osmolytes via an organic osmolyte channel that may be a volume-sensitive anion channel (*see* Strange & Jackson, 1995). For recent reviews on cell volume regulation, *see* Spring & Hoffmann (1992), Hoffmann, Simonsen & Lambert (1993), Strange (1994), and Hoffmann & Dunham (1995).

The K^+ channel involved in the RVD response has often been presumed to be a Ca^{2+}-activated K^+ channel, and the Cl^- (and organic osmolyte) channel(s) have also been presumed to be activated by Ca^{2+}, directly or perhaps indirectly, thus implying a key role for Ca^{2+} signaling in the activation of the RVD response. In accordance with such a role, Ca^{2+} signaling following hypotonic exposure has been demonstrated in several cell types (for refs. *see below*). The mechanism underlying the swelling-induced Ca^{2+} signal appears, however, to vary between different cell types. In most cell types Ca^{2+} influx from the extracellular medium has been found to play a key role for the swelling-induced Ca^{2+} signal. In these cell types the RVD response was usually found to be dependent upon the presence of external Ca^{2+}, although in some cell types only partially (for refs. *see below*). In some of the cell types the swelling-

induced Ca^{2+} entry has been proposed to be augmented by release of Ca^{2+} from internal stores, e.g., in human intestinal cells (Hazama & Okada, 1990), in renal A6 cells (Urbach, Ó'Cróinín & Harvey, 1995), and in HeLa cells (Sardini, Sepulveda & McNaughton, 1995).

In some cell types, on the other hand, the RVD response has been demonstrated to be essentially unaffected by the presence or absence of extracellular Ca^{2+} (for refs. *see below*). In those cell types any putative Ca^{2+} signal involved in the activation of the RVD response would necessarily be caused by Ca^{2+} release from internal stores, with Ca^{2+} entry playing at most a minor role. In a few cell types, finally, the RVD response has been reported not to be associated with any changes in $[Ca^{2+}]_i$. These include human peripheral blood lymphocytes (Grinstein & Smith, 1990; Ross & Cahalan, 1995; but *see* Schlichter & Sakellaropoulos, 1994), mouse splenocytes (Ross & Cahalan, 1995), and Ehrlich cells (*see below*). For reviews concerning the role of Ca^{2+} in cell volume regulation and for references, *see* Pierce & Politis (1990), McCarty and O'Neil (1992), Foskett (1994), and Hoffmann and Dunham (1995).

A few recent studies have concluded that although an increase in $[Ca^{2+}]_i$ could be detected after hypotonic cell swelling, this rise in $[Ca^{2+}]_i$ did not appear to be a prerequisite for the initiation of the RVD response. A swelling-induced increase in $[Ca^{2+}]_i$ was found in rabbit proximal convoluted tubule cells, both in the presence and absence of external Ca^{2+}. This increase was, however, postulated not to be required for a normal RVD response, since the RVD response, also in the absence of external Ca^{2+}, was unaffected by TMB-8, a reported inhibitor of Ca^{2+} release from intracellular stores (Beck et al., 1991; Breton et al., 1992; but *see* also Terreros & Kanli (1992) and Tinel, Wehner & Sauer (1994)). In salivary acinar cells Foskett et al. (1994) found that cell swelling resulted in an increase in $[Ca^{2+}]_i$ both in the presence and in the absence of external Ca^{2+}, but concluded that the RVD response was not dependent upon this increase in $[Ca^{2+}]_i$ since depletion of intracellular Ca^{2+} stores, by addition of a Ca^{2+}-mobilizing agonist or of ionomycin in the absence of extracellular Ca^{2+}, abolished the swelling-induced increase in $[Ca^{2+}]_i$ without any effect on the RVD response. Moreover, Montrose-Rafizadeh and Guggino (1991) in a study on rabbit medullary thick ascending limb cells found an influx-dependent increase in $[Ca^{2+}]_i$ after cell swelling, but likewise concluded that this increase was not required for a normal RVD response, because the RVD response could be measured in the absence of extracellular Ca^{2+}, under conditions where no increase in $[Ca^{2+}]_i$ could be detected. According to this study, however, the resting level of $[Ca^{2+}]_i$ could be significant, since a reduction of $[Ca^{2+}]_i$ was found to inhibit the RVD response. In vascular endothelial cells Szücs et al. (1996) have reported that the volume-sensitive Cl^- channels are not modulated by changes in $[Ca^{2+}]_i$, but that their activation requires a permissive intracellular Ca^{2+} concentration. Overall, therefore, the role of Ca^{2+} in the initiation of the RVD response seems unclear.

In the case of the Ehrlich ascites tumor cell, a model for the activation mechanism for the RVD response has been proposed, involving Ca^{2+} entry via stretch-activated Ca^{2+} channels as well as Ca^{2+} mobilization via swelling-induced activation of phosphoinositidase C and ensuing $Ins(1,4,5)P_3$ production (*see* Hoffmann, Simonsen & Lambert, 1993). As mentioned above, however, a significant increase in $[Ca^{2+}]_i$ after hypotonic cell swelling could not be detected in fura-2-loaded Ehrlich cells (Thomas-Young, Smith & Levinson, 1993; Harbak & Simonsen, 1995). In these studies, though, the average Ca^{2+} signal was recorded from cell clusters or from cells in suspension, and hence the absence of a detectable Ca^{2+} signal would neither exclude asynchronous Ca^{2+} signaling in the cell population nor 'localized' increases in cytosolic $[Ca^{2+}]_i$.

In previous work from this laboratory (Hoffmann, Simonsen & Lambert, 1984; Hoffmann, Lambert & Simonsen, 1986) it has been shown that an increase in $[Ca^{2+}]_i$ following addition of the Ca^{2+} ionophore A23187 induces a KCl loss from the cells in isotonic medium and accelerates the RVD response in hypotonic medium, and at the time these findings were taken to suggest a role for Ca^{2+} in the activation of the KCl loss during RVD. A similar acceleration of the RVD response has in later experiments been seen after addition of Ca^{2+}-mobilizing agonists such as thrombin and bradykinin (Simonsen et al., 1990; *see* Hoffmann et al., 1993). The RVD response could also be accelerated by a sudden increase in the external Ca^{2+} concentration, and this was taken as further circumstantial evidence for the involvement of the Ca^{2+}-dependent K^+ channel in the volume recovery in hypotonic media (Hoffmann et al., 1984). Furthermore, the inhibition of the RVD response by quinine was at the time taken to indicate the involvement of Ca^{2+}-activated K^+ channels (Hoffmann et al., 1984, 1986). Ca^{2+}-activated, inwardly rectifying K^+ channels were subsequently demonstrated in excised inside-out patches from Ehrlich cells, and in cell-attached patches after addition of the Ca^{2+} ionophore A23287 (Christensen & Hoffmann, 1992). Finally, the finding that pimozide and other calmodulin antagonists strongly inhibited both the volume recovery following hypotonic cell swelling and the ionophore A23187-induced KCl loss in isotonic medium, supported the notion that $[Ca^{2+}]_i$ played a key role in the activation mechanism for the RVD response (Hoffmann et al., 1984, 1986). In the Ehrlich cell the RVD response is unaffected by the presence or absence of external Ca^{2+}, and on this basis it was proposed (Hoffmann et al., 1984), that swelling-induced release of Ca^{2+}

from internal stores could account for the activation of K^+ and Cl^- channels, or that perhaps the Ca^{2+} sensitivity of the channels might be increased after cell swelling. It was later in preliminary studies reported that PtdIns(4,5)P_2 showed a rapid decrease after hypotonic exposure (Christensen et al., 1988), and that Ins(1,4,5)P_3 showed a transient increase during RVD (Christensen et al., 1988; Svane et al., 1990; Svane & Hoffmann, 1991), suggesting activation of phosphoinositidase C during RVD with ensuing Ca^{2+} release from internal stores.

To assess the putative role of Ca^{2+} in the activation of the RVD response in Ehrlich cells we have explored: (i) whether a genuine increase in cellular Ins(1,4,5)P_3, that would provide evidence for volume-induced Ca^{2+} mobilization, can be detected after hypotonic cell swelling, (ii) the occurrence of Ca^{2+} signaling during RVD in a suspension of fura-2-loaded cells, and in single fura-2-loaded cells monitored by fluorescence-ratio imaging, (iii) the volume-induced K^+ loss in the presence and absence of external Ca^{2+}, (iv) the effect on the RVD response of loading the cells with the Ca^{2+} chelator BAPTA, and (v) the effect of K^+ channel blockers reported to inhibit the K^+ loss induced by Ca^{2+}-mobilizing agonists in Ehrlich cells on the volume-induced K^+ loss. The data obtained provide strong evidence against a central role for raised $[Ca^{2+}]_i$ in the normal RVD response.

Part of the present data have previously been presented in preliminary form (Simonsen et al., 1990; Jørgensen, Lambert & Hoffmann, 1994; Harbak & Simonsen, 1995).

Materials and Methods

Cell Suspension

The Ehrlich ascites tumor cell line (hyperdiploid strain) was maintained in female NMRI mice by weekly intraperitoneal inoculation. The mice were killed by cervical dislocation, and the cells harvested and washed with standard medium by gentle centrifugation (for details *see* Hoffmann et al., 1986). The cells were suspended to cytocrit 4% in standard medium and preincubated for 15–30 min. During this period loading of the cells with fura-2 or with BAPTA was initiated when appropriate (*see below*). For experiments where Cl^- was replaced by nitrate, the cells were preincubated for about 15 min in standard medium, and then washed once, resuspended and incubated in the nitrate medium for 15–30 min in order to allow for exchange of intracellular Cl^- with nitrate. All experiments were conducted at 37°C.

Media

Standard medium (300 mOsm) contained (with concentrations given in mM): 150 Na^+, 5 K^+, 1 Mg^{2+}, 1 Ca^{2+}, 150 Cl^-, 1 SO_4^{2-}, 1 HPO_4^{2-}, 3.3 MOPS, 3.3 TES, 5 HEPES, pH 7.4. In nitrate medium Na^+, K^+, and Ca^{2+} nitrates were substituted for NaCl, KCl, and $CaCl_2$, respectively. Ca^{2+}-free medium was prepared by omission of $CaCl_2$ and addition of EGTA (1 or 2 mM, as indicated). Low Ca^{2+}-medium (100 μM free Ca^{2+}) was prepared using 2-mM EGTA as Ca^{2+} buffer with addition of $CaCl_2$ as calculated by the use of EQCAL software (BioTools, 1988). High Ca^{2+} medium (3 mM) was prepared as the standard medium except that $CaCl_2$ was added to a total concentration at 3 mM. Hypotonic media were prepared by appropriate dilution of the standard medium with buffered water, to reduce the osmolarity as indicated. Buffered water is distilled water containing buffers in concentrations as in the standard medium and adjusted to pH 7.4. The buffered water was usually Ca^{2+}-free, except where indicated (*see* Table 1 and legends to figures). K^+ *equilibrium medium* contained 50 mM K^+ and was prepared by substitution of the appropriate amount of KCl for NaCl. In experiments with addition of Ba^{2+} to the cell suspension the incubation medium was sulfate-free, $MgCl_2$ being substituted for $MgSO_4$. The incubation medium contained dialyzed albumin (10 mg/ml) in the experiments with measurements of cellular inositol phosphate or K^+ content (*see below*).

Reagents

All reagents were analytical grade and unless otherwise indicated obtained from Merck or Sigma. Ionomycin, thrombin, bradykinin, clotrimazole, kaliotoxin and poly-L-lysine were obtained from Sigma. Charybdotoxin was purchased from Latoxan (Rosans, France). Margatoxin and TEA were obtained from Alomone Labs (Jerusalem, Israel). Fura-2-AM, Fura-2-P (pentapotassium salt), and BAPTA-AM were obtained from Molecular Probes (OR, USA). [2-^{3}H]*myo*-inositol (TRK.911) was obtained from The Radiochemical Centre, Amersham (Bucks, UK). Di-n-butyl phthalate, bis(2-ethylhexyl)phthalate, and perchloric acid (60% w/w) were obtained from Merck. Bovine serum albumin, fraction V (BSA) was obtained from Sigma, and extensively dialyzed against distilled water where indicated. Fura-2-AM was added from a 1-mM stock solution in dry DMSO, the final DMSO concentration being 0.1 or 0.2%. BAPTA-AM was added from a 10-mM stock solution in dry DMSO, with a final DMSO concentration at 0.5%. Fura-2-P was added from a 1-mM stock solution in distilled water. Ionomycin was added from a 1-mM stock solution in ethanol, the final ethanol concentration being 0.2%. Clotrimazole was added from a 10-mM stock solution in dry DMSO. Thrombin was added from a 1,000 U/ml stock solution in water, containing the buffer salts from the thrombin preparation, and bradykinin added from a 1-mM stock solution in distilled water. The peptide toxins kaliotoxin and margatoxin were added from stock solutions at 20 μM and 2 μM, respectively, in 100 mM NaCl, 1 mM EDTA, 1 mg/ml BSA, 10 mM Tris, pH 7.5. Charybdotoxin was added from a 5 μM stock solution in standard incubation medium with BSA, 1 mg/ml. The control groups received addition of buffer (vehicle) alone. The stock solutions were stored in aliquots at −20°C. The charybdotoxin stock solution was freshly prepared or stored at 4°C for a week.

Measurement of Cellular Inositol Phosphate Content

Ehrlich cells were labeled in vivo with [2-^{3}H]*myo*-inositol by intraperitoneal injection (0.3 to 0.5 mCi) in tumor-bearing mice 18 to 22 hr before harvesting the cells. After collecting the ascites fluid with 10-fold dilution in standard medium the cells were sedimented, washed once, and preincubated for 20 min at 5% cytocrit in standard medium, 37°C. Parallel aliquots of the cell suspension were subsequently transferred to a multihead magnetic stirrer and incubated at 37°C. All media contained dialyzed albumin (10 mg/ml) and silanized vials were used throughout, in order to minimize the content of inositol phosphates present in the extracellular medium (*see* Results).

Serial aliquots of the cell suspension and of the extracellular

medium (supernatant after brief centrifugation (10 sec, 17,000 × *g*)) were transferred to ice-cold PCA (final concentration 0.5 M), extracted for 20 min on ice with frequent mixing, and the supernatant neutralized with tri-n-octylamine-freon (Sharpes & McCarl, 1982). The neutralized PCA extracts were subsequently analyzed by anion-exchange chromatography, either by HPLC using a Mono-Q HR 5/5 anion-exchange column (Pharmacia Biotech, Uppsala, Sweden), or usually by small-column, ambient-pressure anion-exchange chromatography using glass columns packed with BioRad AG 1-X8 formate resin, and stepwise elution with increasing concentrations of ammonium formate in 0.1 M formic acid. To all samples was added Ca^{2+}-chelator (EDTA, 4 mM) and $InsP_6$ hydrolysate. ^3H activity was measured by liquid scintillation counting, using Hionic Fluor (Packard) as scintillation fluid. For details *see* Christensen & Harbak (1990) and Christensen, Harbak & Simonsen (1994). Following hypotonic or isotonic dilution of the cell suspension the sampling volume was increased in proportion to the dilution such that all samples nominally represent the same number of cells.

The cell content (cpm) of [^3H] inositol phosphates was calculated from the total content measured in the cell suspension by subtraction of the content in the extracellular medium, as measured in duplicate samples of the supernatant taken before and again after the hypotonic dilution.

Measurement of Cell K^+ Content

Serial aliquots (500 to 1,000 μl) of the cell suspension (about 5% cytocrit) were layered on top of an oil layer (200 μl of a mixture of di-n-butyl phthalate and bis(2-ethylhexyl) phthalate (12:8, w/w), with the density (nominally 1.020 g/ml) between that of the suspending medium and of the cells), and centrifuged briefly (10 sec, 17,000 × *g*) in an Eppendorf type microfuge (Ole Dich, Copenhagen, Denmark). The cell pellet below the oil layer was lysed in distilled water and precipitated with PCA (final concn. 0.3 M). The K^+ concentration in the PCA supernatant was measured by emission flame photometry, using Li^+ as internal standard (Radiometer FLM3 Flame Photometer). The cell K^+ content (μmol/ml original cell volume) was calculated with reference to the original volume of cells, as estimated from duplicate measurements of the cytocrit value before the hypotonic/isotonic dilution, and is given relative to the initial cell K^+ content within the group (about 110 to 120 μmol/ml cells). No correction for trapped extracellular volume in the cell pellet below the oil was applied. The trapped volume amounts to about 0.15 ml/ml packed cells, and hence the correction would amount to less than 1% of the K^+ signal.

Cell Volume Measurements

Cell volume was determined by electronic cell sizing using a Coulter counter model ZB equipped with a Coulter Channellyzer (C-1000) as described by Hoffmann et al. (1984). Briefly, an aliquot of the cell suspension (4% cytocrit) was diluted 400-fold into filtered (pore size 0.45 μm) experimental medium resulting in an experimental cell density at about 90,000 cells per ml (cytocrit ~0.01%). The cell counting proceeded until 1,000 cells were recorded in the modal channel (or 4,000 cells in the experiment presented in Fig. 5). The mean cell volume (in arbitrary units) was calculated as the median of the cell volume distribution curves. By the use of polystyrene latex beads (12.9 μm diameter, Coulter Electronics) as standards the absolute cell volumes in fl (i.e., 10^{-15} liter) could be obtained. During regulatory volume decrease (RVD) in hypotonic media the initial rate of the RVD response was calculated as the rate of cell shrinkage (Δfl/min) between 1 and 3 min after the reduction in osmolarity, using linear regression. The volume recovery (*R*) after 5 min (*see* Fig. 9) was calculated as $R = (V_{max} - V_5)/(V_{max} - V_0)$, where V_0 is the initial cell volume, V_{max} the maximal cell volume, and V_5 the cell volume recorded 5 min after the hypotonic exposure.

Loading of Ehrlich Cells with Fura-2

Ehrlich cells were loaded with the Ca^{2+}-sensitive fluorescent probe fura-2 by incubation of the cell suspension (cytocrit 0.4%) for 20 min at 37°C in standard medium containing fura-2-AM, 2 μM (single cell studies) or 1 μM (cell suspension measurements in this study), and BSA (2 mg/ml). In early cell suspension measurements the cells were loaded with 5-μM fura-2-AM at 2% cytocrit. After the incubation the cells were washed once with standard medium containing BSA (2 mg/ml) and once with the appropriate experimental medium, and finally resuspended in the appropriate medium at a cytocrit of 0.4% (single cell experiments) or 5% (cell suspension measurements). To avoid leakage of the dye, the cell suspension was kept under gentle stirring at room temperature (about 20°C).

Loading of Ehrlich Cells with BAPTA

Ehrlich cells (cytocrit 0.4%) were loaded with BAPTA by incubation with 50-μM BAPTA-AM in standard medium, or in K^+ equilibrium medium, with BSA (2 mg/ml) for 35 min at 37°C, and finally washed twice and resuspended as described for fura-2-loading (*see* Jørgensen, Lambert & Hoffmann, 1996).

Monitoring the Free Intracellular Ca^{2+} Concentration

In Cell Suspensions

This was done essentially as described in Jørgensen et al. (1996). Briefly, samples of the suspension of fura-2-loaded cells were diluted 10-fold in the experimental medium at 37°C. The ratio (f340/f380) of the fluorescence intensities with excitation at 340 and 380 nm, respectively, was obtained with about 7-sec intervals. The fura-2-loaded cells slowly leak fura-2 to the medium, especially at 37°C. As fura-2 in the Ca^{2+}-containing medium is saturated with Ca^{2+}, this leak gives rise to a slow, steady increase in the fluorescence-ratio signal from the cell suspension. No attempt was made to correct for this increasing background.

In Single Cells

$[Ca^{2+}]_i$ was determined in single fura-2-loaded cell using fluorescence microscopy with digital image processing as described in Jørgensen et al. (1996). The cell suspension (2 to 4 ml, cytocrit 0.3%) was placed in a chamber thermostatically controlled at 37°C; the coverslips used in these experiments were coated with poly-L-lysine (25 mg/ml) to promote good cell attachment. Fluorescence recordings were initiated after about 5 min. The fluorescence ratio (340 nm/380 nm) was recorded in the central region of the cell, neglecting the periphery to avoid possible artifacts in recording the ratio close to the cell membrane, caused by changes in the cell shape and volume in the time interval between the collection of the 340 nm and the 380 nm image. The fluorescence ratio-values were collected and stored with a time resolution of approximately 2.5 sec.

The field of view contained on average 12 individual cells. In single cell studies all cells examined showed a good, stable fluores-

cence intensity. The few cells that showed a weak intensity and/or leakage of the dye were ignored. Only healthy looking cells with a resting level of $[Ca^{2+}]_i$ in the normal range (around 60 nM in standard medium, *see* Jørgensen et al., 1996) were included in the study.

The cells were exposed to hypotonicity (final osmolarity 50%) by addition of one volume of buffered water, containing the appropriate Ca^{2+} concentration. In all experiments Ca^{2+}-mobilizing agonists (bradykinin and/or thrombin) were added (by pipette, as 10 μl stock solution) at the end of the experiment as a control of normal cell response and cell viability.

Calibration of the Fura-2 Fluorescence Signal

The 340 nm/380 nm fluorescence ratio was converted to a Ca^{2+} concentration as described by Grynkiewicz, Poenie & Tsien (1985) using in vitro calibration as described in detail in Jørgensen et al. (1996). The data are, however, presented mainly as the measured 340 nm/380 nm fluorescence ratio, rather than as the calculated $[Ca^{2+}]_i$ value because of calibration problems which may arise when the cells swell following exposure to hypotonic media, with resulting changes in ionic strength and probably cytosolic viscosity (*see* Results; for discussion of potential artifacts in measurements using fluorescent probes see also Negulescu & Machen (1990)).

Statistical Evaluation

The values are given as the mean ± SE of the mean, except where otherwise indicated. The number of experiments (n) is indicated in parentheses. Statistical significance was evaluated by the use of Student's t-test.

Abbreviations

AM: Acetoxymethyl ester; BSA: Bovine serum albumin; DMSO: Dimethylsulfoxide; EDTA: Ethylenediamine-tetraacetic acid; EGTA: Ethylene-glycol-bis-(β-amino-ethyl-ether)N,N,N′,N′-tetraacetic acid; HEPES: N-2-hydroxyethylpiperazine-N′-2-ethane sulfonic acid; LTD_4: Leukotriene D_4; MOPS: 3-(N-morpholino) propane sulfonic acid; PCA: Perchloric acid; RVD: Regulatory Volume Decrease; TEA: Tetraethylammonium chloride; TES: N-tris-(hydroxymethyl)methyl-2-aminoethane sulfonic acid.

Results

Effect of Hypotonic Exposure on the Cell Content of ^{3}H-labeled Inositol Phosphates

The effect of hypotonic exposure of Ehrlich cells on the cell content of ^{3}H-labeled inositol phosphates was investigated in order to explore whether activation of phosphoinositidase C is involved in the activation of RVD response. Figure 1 (panel A) demonstrates that cell swelling (osmolarity reduced to 50%) has no effect on the cell content of ^{3}H-labeled inositol phosphates ($InsP_2$, $InsP_3$, and $InsP_4$) as measured by small-column anion-exchange chromatography. In a parallel experiment the cell content of [^{3}H]inositol phosphate was analyzed by HPLC. The results showed that the cell content of neither $Ins(1,4,5)P_3$ nor $Ins(1,3,4)P_3$ was increased following the hypotonic dilution (*not illustrated*).

The cell content of [^{3}H]inositol phosphates is corrected for the content in the extracellular medium. The

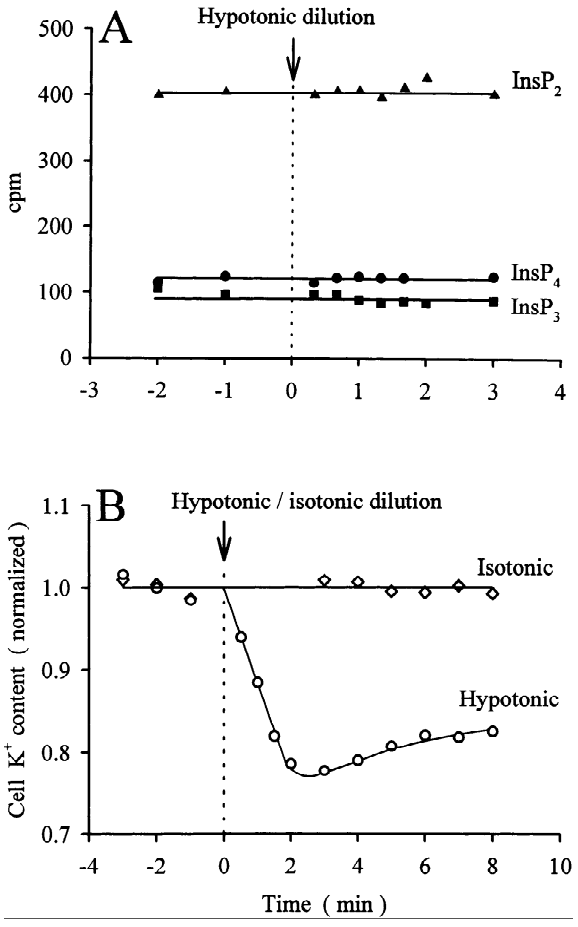

Fig. 1. Time course of cell content of ^{3}H-labeled inositol phosphates and of cell K^+ content following hypotonic exposure. *A:* A suspension of cells labeled in vivo with [2-^{3}H]*myo*-inositol was at zero time (arrow) diluted with 1.5 volumes 1/6 strength incubation medium, such as to reduce the osmolarity by 50%. A parallel group (control) was diluted with the same volume of isotonic 1/1 standard medium (*not illustrated*). All media contained dialyzed albumin (10 mg/ml). The samples all nominally represent the same number of cells. Analysis of [^{3}H] inositol phosphate content was by small-column anion-exchange chromatography. The cell content of [^{3}H]inositol phosphates is corrected for the content in the extracellular medium. Notice that $InsP_4$ is presumably significantly overestimated due to the presence of ATP with aberrant ^{3}H-labeling in the PCA-cell extract (*see* Christensen et al., 1994). *B:* In a control experiment with unlabeled cells (from the same inoculation) the cell K^+ content was followed with time in parallel groups (each in duplicate) after hypotonic and isotonic dilution, respectively, with the same volume and using the same diluting media (containing dialyzed albumin) as used in the upper panel. All samples nominally represent the same number of cells. The ordinate gives the cell K^+ content relative to the initial value. The values presented are the mean values of the duplicate groups. Typical experiment. Two further experiments, also with parallel controls with unlabeled cells, gave similar results.

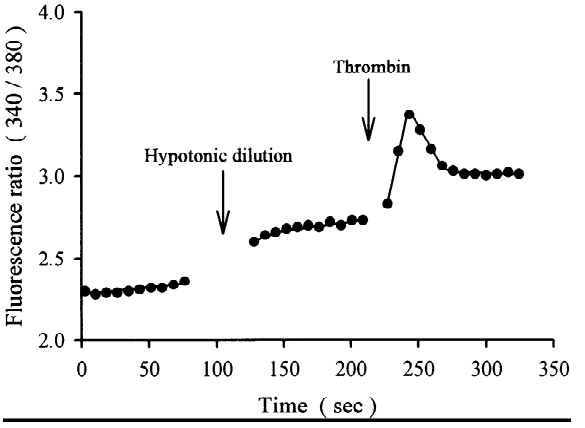

Fig. 2. Effect of hypotonic exposure on the fluorescence signal recorded from a suspension of cells loaded with the Ca^{2+}-probe fura-2. The fluorescence signal was recorded from a suspension of fura-2-loaded cells in standard incubation medium (1-mM Ca^{2+}) at about 0.5% cytocrit. At the time indicated by the first arrow the cell suspension was diluted with 1 volume of buffered water, such as to reduce the osmolarity by 50%. The second arrow marks the addition of thrombin (1 U/ml). The ordinate gives the ratio of the fluorescence emission obtained with excitation at 340 nm and 380 nm, respectively. Two other experiments gave similar results. In 3 early experiments using a slightly modified protocol where the fura-2-loaded cells were stored on ice, the cells suspension was in parallel runs diluted with either buffered water or isotonic medium. Following the hypotonic dilution a slight upward shift of the fluorescence ratio, similar to that seen in the figure, was observed. This shift was absent following isotonic dilution (*not illustrated*). The slight upward shift in hypotonic media is presumably artifactual and does not represent a genuine increase in $[Ca^{2+}]_i$ (*see* text).

extracellular content of inositol phosphates is dominated by InsP, $InsP_2$, and $InsP_3$ (predominantly the $Ins(1,4,5)P_3$ isomer). The extracellular content of inositol phosphate, when taken relative to the total content in the cell suspension, amounts to about 30% in the case of $InsP_3$, with smaller percentages being found for the other inositol phosphates. In one experiment this extracellular [^{3}H] inositol phosphate content was followed with time in parallel samples of the supernatant taken 20 sec after each corresponding sample of the cell suspension. The extracellular content was constant with time, but was shifted slightly upwards in response to the hypotonic dilution.

In Fig. 1 (panel B) the cell K^+ content was monitored in a parallel group of cells from the same inoculation, and exposed to the same diluting media as used in the experiment shown in panel A. The cells demonstrated a typical RVD response shown as a decrease in the cell K^+ content within the first few minutes following the hypotonic dilution. In the present experiment the K^+ loss was recorded in the presence of dialyzed albumin (10 mg/ml). The swelling-induced K^+ loss is, however, unaffected by the presence of dialyzed albumin when compared to the K^+ loss recorded in previous experiments in the absence of albumin (*not illustrated*). These findings show that activation of phosphoinositidase C after cell swelling in Ehrlich cells is not a prerequisite for a normal RVD response, arguing against a role for the phosphoinositidase C signaling pathway in the activation of the RVD response.

Effect of Hypotonic Exposure upon the Fluorescence Signal Recorded from a Suspension of Fura-2-loaded Cells

Figure 2 shows the effect of hypotonic dilution on $[Ca^{2+}]_i$ measured in a suspension of fura-2-loaded Ehrlich cells. Whereas a typical Ca^{2+}-signal could be elicited by addition of the Ca^{2+}-mobilizing agonist thrombin either in the hypotonic medium (*see* Fig. 2), or when thrombin was added with the buffered water, i.e., at the time of hypotonic exposure (*not illustrated*), reduction of the osmolarity to 50% produces only a slight parallel shift upwards of the fluorescence ratio (340 nm/380 nm). The origin of this minor shift is unclear. No upward shift was observed following isotonic dilution (*not illustrated*). Although in the hypotonic medium a small subgroup of cells responding with a transient increase in $[Ca^{2+}]_i$ (*see* Table 1) would contribute to the observed upward shift in the fluorescence ratio, the osmotically induced cell swelling could also affect the recorded fluorescence-ratio in several other ways apart from changes in $[Ca^{2+}]_i$, e.g., by changes in light scattering and UV light absorbance at 340 and 380 nm, in cytosolic viscosity, ionic strength and activity coefficients, and hence in Ca^{2+}-binding and fluorescence spectra of fura-2. The slight apparent increase in $[Ca^{2+}]_i$ observed may therefore be artifactual and perhaps due mostly to a swelling-induced decrease in cytosolic viscosity, which should decrease the fluorescence intensity predominantly at longer wavelengths (Roe, Lemasters & Herman, 1990; Poenie, 1990), with resulting increase in the calculated fluorescence ratio. A swelling-induced artifactual increase in $[Ca^{2+}]_i$ attributed to a decrease in the fura-2 fluorescence at 380 nm, unrelated to Ca^{2+} binding, has been demonstrated by Botchkin and Matthews (1993) in the case of single rat retinal pigment epithelial cells exposed to hypotonicity. Moreover, leakage of fura-2 following the hypotonic exposure from some of the cells into the Ca^{2+}-containing medium could conceivably contribute to an upward shift. Therefore, the slight upward shift quite possibly does not reflect a genuine increase in $[Ca^{2+}]_i$ in the main fraction of the cell population.

$[Ca^{2+}]_i$ Profiles in Fura-2-loaded Single Cells During Hypotonic Cell Swelling

The above findings provide no evidence of Ca^{2+}-signaling induced by cell swelling. On the other hand,

Table 1. Effect of hypotonic exposure upon $[Ca^{2+}]_i$ - single cell experiments

Medium	Total number of experiments*	Experiments with one or more responding cells	Total number of responding cells	Responding cells in % of total number of cells
3 mM Ca^{2+}	11	4	8	6%
1 mM Ca^{2+}	55	25	39	6%
100 μM free Ca^{2+} (buffered with 2 mM EGTA)	14	1	1	1%
Ca^{2+}-free (2 mM EGTA)	18	1	2	1%

* Each experiment included on the average 12 cells in the field of view. This number of cells was used in the estimate of the percentage of responding cells (column 5).
The fura-2-loaded cells were washed once with standard medium containing BSA, and once with isotonic medium with the Ca^{2+} concentration indicated (*see* column 1), and subsequently preincubated for 10–30 min (cytocrit 0.4%). The cell suspension was transferred to the microscope chamber, and the cells after about 5 min exposed to hypotonicity (final osmolarity 50%) by addition of an equal volume of buffered water, containing the appropriate Ca^{2+} concentration. The fluorescence-ratio (340 nm/380 nm) was followed with time in single cells by video imaging (*see* Materials and Methods) until 12 min after the hypotonic exposure. In experiments with responding cells, only one or a few of the cells in the field of view showed a response, i.e., a transient increase in the fluorescence ratio.

the findings would not exclude the possible occurrence of asynchronous Ca^{2+} signaling in the cell population following hypotonic exposure, nor the occurrence of 'localized', swelling-induced increase in $[Ca^{2+}]_i$ by Ca^{2+} entry or by release of Ca^{2+} from subcellular compartments. To explore these possibilities, studies of $[Ca^{2+}]_i$ in single cells using fluorescence-ratio imaging were undertaken. Table 1 shows data from experiments in which $[Ca^{2+}]_i$ was monitored in single, fura-2-loaded cells using fluorescence-ratio imaging. The cells were preincubated for 10–30 min at the Ca^{2+} concentration indicated. The resting $[Ca^{2+}]_i$ level was estimated at 59 nM and 23 nM in medium with 1 nM Ca^{2+} and in Ca^{2+}-free medium (2-mM EGTA), respectively (*see* Jørgensen et al., 1996). The cells were exposed to hypotonicity (osmolarity reduced by 50%) by addition of one volume of buffered water, also containing the Ca^{2+} concentration indicated. The data show that a response, i.e., an increase in the 340 nm/380 nm fluorescence ratio reflecting an increase in $[Ca^{2+}]_i$, could not be detected in most experiments. An increase in $[Ca^{2+}]_i$ in one or more cells in the field of view (on the average containing 12 cells, *see* legend to Table 1) was detected in only about 40% of the experiments in media containing 1 or 3 mM Ca^{2+} (55 and 11 experiments, respectively), in only one of the 14 experiments in medium with a low Ca^{2+}-concentration (100-μM free Ca^{2+}), and in only one of the 18 experiments in Ca^{2+}-free medium (with 2-mM EGTA). Moreover, in experiments with responding cells only a single or a few of the cells examined showed a response. In Ca^{2+}-containing media only about 6% of the total number of the cells examined showed a response, and in medium with a low Ca^{2+} concentration (100-μM free Ca^{2+}) or in Ca^{2+}-free medium (2-mM EGTA) only about 1% of the cells responded with an increase in $[Ca^{2+}]_i$. In this type of single cell experiments it is usually found that not all the cells in the field of view respond to a specific stimulation. After addition of a Ca^{2+}-mobilizing agonist (bradykinin, thrombin, or LTD_4), however, about 70% of the cells showed an increase in $[Ca^{2+}]_i$ (*not illustrated*). In this context it may be noted that analysis of the cell volume distribution curves recorded from the Coulter Channellyzer following hypotonic exposure (*see* Fig. 5) gave no indication of the presence of subgroups of responding and nonresponding cells, respectively. No evidence of 'localized' increases in $[Ca^{2+}]_i$ was detected within the spatial resolution of the present study.

Figure 3 shows selected examples of single-cell $[Ca^{2+}]_i$ profiles in the subgroup of cells (6% of total) responding with $[Ca^{2+}]_i$ transients during hypotonic exposure in medium containing 1 mM Ca^{2+}. It can be seen that the $[Ca^{2+}]_i$ profile after hypotonic treatment—in those cells eliciting $[Ca^{2+}]_i$ transients—is quite variable. All the detected responses are transient, i.e., a rapid increase in $[Ca^{2+}]_i$ followed by a decrease towards the resting level. In the responding cells, however, the time lag after the hypotonic exposure before a response is observed varies from cell to cell, and a few cells even show more than one transient increase in $[Ca^{2+}]_i$. The magnitude of the peak response recorded is also quite variable (*see* Fig. 3). In a few experiments an occasional cell responded to hypotonicity by a sustained increase in $[Ca^{2+}]_i$. These data have been discarded since such cells

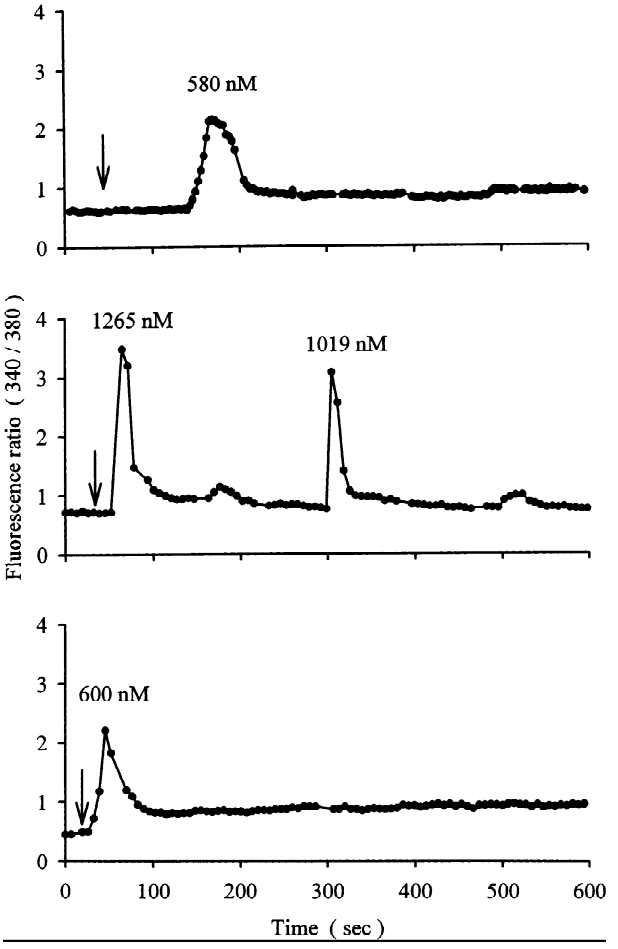

Fig. 3. Selected examples of single cells showing swelling-induced $[Ca^{2+}]_i$ transients. Experimental protocol as in Table 1, using standard medium (1 mM Ca^{2+}). The data show the fluorescence ratio (340 nm/380 nm) as function of time in three responding cells. The cells were exposed to hypotonic medium (50% osmolarity) at the time indicated by the arrow. The inserted numbers are cytosolic Ca^{2+} concentrations, estimated from the fluorescence-ratio peak value (*see* Materials and Methods). The data are selected examples from the subgroup of 39 responding cells out of the 660 cells investigated (*see* Table 1).

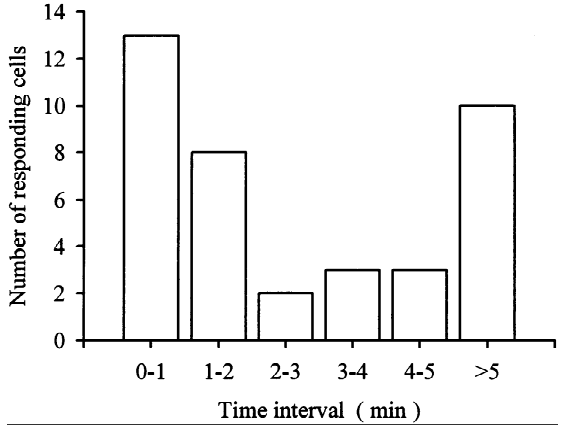

Fig. 4. Time lag for the swelling-induced $[Ca^{2+}]_i$ transients in the responding cells. Experimental protocol as in Fig. 3. The number of responding cells (ordinate) is the number of cells showing a transient increase in $[Ca^{2+}]_i$ (measured as an increase in the 340 nm/380 nm fluorescence ratio) after the hypotonic exposure. The time interval (abscissa) is the time lag before the $[Ca^{2+}]_i$ peak was recorded for the total of 39 responding cells, with the data being subdivided into 1-min intervals. For the occasional cell showing two $[Ca^{2+}]_i$ peaks (*see* Fig. 3), only the time lag for the first $[Ca^{2+}]_i$ peak was included in the figure.

are interpreted as cells suffering damage by the hypotonic exposure.

Figure 4 shows the distribution of the time lag for the swelling-induced $[Ca^{2+}]_i$ transients in the subgroup of responding cells (6% of total at 1-mM external Ca^{2+}) and illustrates the variability of the response. It should be noted that the increase in $[Ca^{2+}]_i$ in about half of the responding cells occurs later than 2 min after the hypotonic exposure.

Thus, very few cells only respond to hypotonic exposure with an increase in $[Ca^{2+}]_i$, and in those cells that do respond the time lag between the hypotonic stimulus and the increase in $[Ca^{2+}]_i$ is quite variable. These findings are consistent with the measurements from cell suspensions where no evidence of Ca^{2+}signaling was found (*see* Fig. 2).

No Effect of Fura-2-loading on the RVD Response

Fura-2-loading of the cells did not significantly affect the rate of the RVD response. Monitoring the cell volume with the Coulter counter following dilution into hypotonic (150 mOsm) standard medium, the rate of the RVD response in fura-2-loaded cells was not significantly different from that in parallel groups of control cells (*not illustrated*). In three of the four experiments the RVD response was accelerated, and in one experiment inhibited, in the fura-2-loaded cells compared to control cells.

Homogeneity of the RVD Response in the Cell Population

The finding that only a subgroup of the cells responded with $[Ca^{2+}]_i$ transients during RVD prompted an analysis of the homogeneity of the RVD response in the cell population. Figure 5 (panels *A* and *B*) shows the cell volume distribution curves recorded from the Coulter Channellyzer for isotonic control cells (*curve a*) and for cells exposed to hypotonic medium (50% osmolarity), at the time of maximal cell swelling (*curve b*), 3 min after the hypotonic exposure (*curve c*), and when the volume recovery is nearly completed after 10 min (*curve d*). Panel *A* (top left) shows curves recorded in Ca^{2+}-containing medium, and Panel *B* (top right) shows simi-

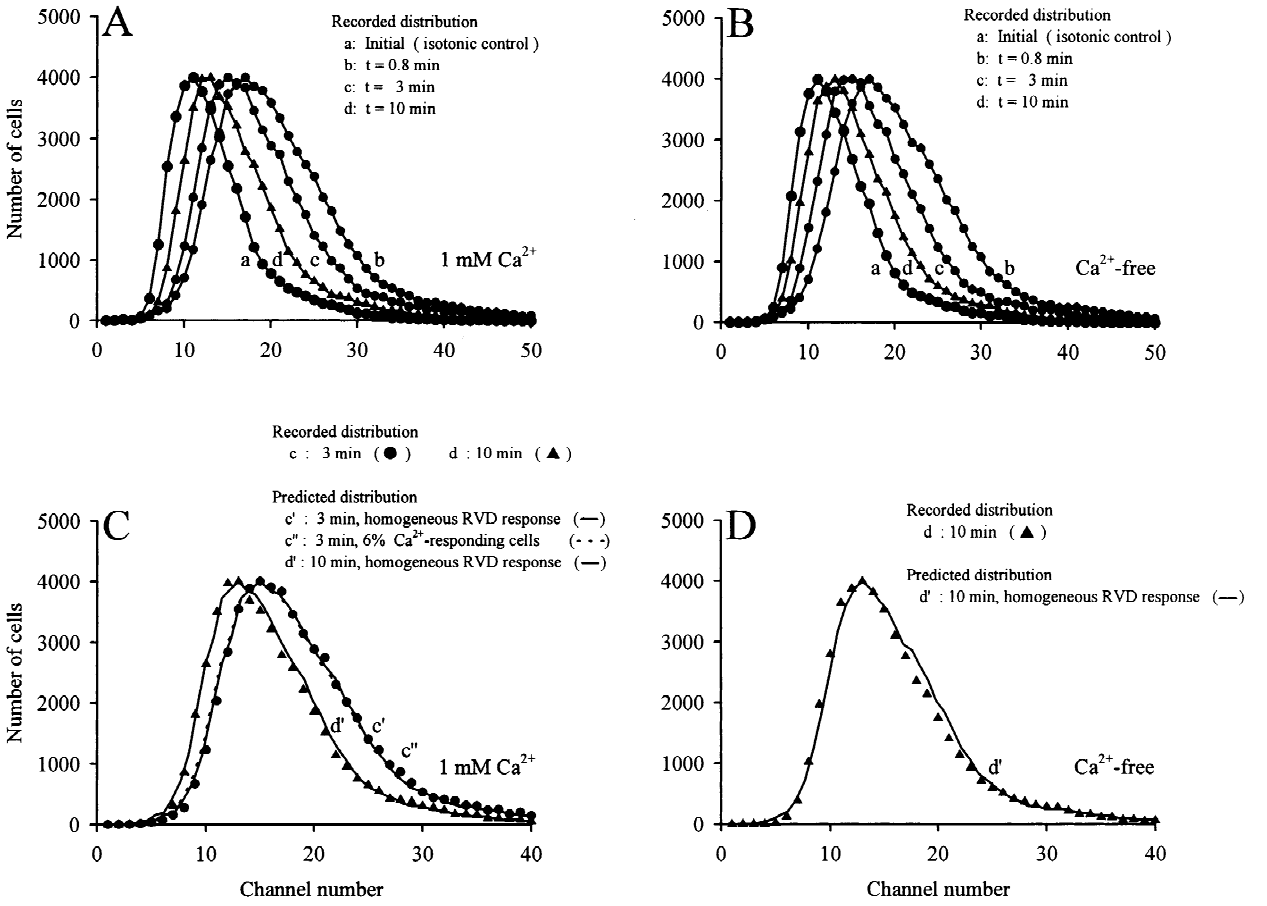

Fig. 5. Cell volume distribution curves following hypotonic exposure in Ca^{2+}-containing and Ca^{2+}-free medium. *A:* The cell suspension (cytocrit 4%) was preincubated for 35 min in standard medium (1-mM Ca^{2+}), and aliquots subsequently diluted 400-fold into standard medium or hypotonic medium (50% osmolarity), 1 mM in Ca^{2+}. Cell volume distribution curves, i.e., number of cells *vs.* channel number, were obtained with a Coulter channelyzer about 1 min after isotonic dilution (*curve a*), and 0.8, 3, and 10 min after the hypotonic exposure (*curves b, c, and d,* respectively). *B:* Parallel group with same protocol, except that dilution was with Ca^{2+}-free media (containing 1-mM EGTA). In *C and D* the data points for the cell volume distribution recorded at $t = 3$ and 10 min are presented again (by their *symbols only*), and compared to predicted cell volume distribution curves (*solid and broken lines*). The predicted curves *c′* and *d′* (*solid lines*) were calculated under the assumption that all swollen cells recover their volume to the same extent, by multiplication of the cell volumes at $t = 0.8$ min (*curve b*) by the ratio between the modal volumes for curves *c* and *b* (*curve c′*, Panel C only), and for curves *d* and *b,* respectively (*curve d′*). *Curve c″* (*broken line,* panel C only) was calculated assuming (*see* text) that a subgroup at 6% of the cells respond with $[Ca^{2+}]_i$ transients and exhibit volume recovery back to the initial, isotonic cell volume within 3 min, and that the remaining 94% of the cells only regulate their volume corresponding to the shift in modal volume within this time interval. For those 6% of the cells with an assumed volume recovery back to initial values, the predicted cell volumes were calculated by multiplication of the cell volumes at $t = 0.8$ min (*curve b*) by the ratio between the modal volumes for curves *a* and *b.* For the remaining 94% of the cells the predicted cell volumes were calculated as for curve *c′*. In one other experiment similar results were obtained.

lar curves recorded in Ca^{2+}-free medium containing 1-mM EGTA. The curves show, as expected, that the cells swell in the hypotonic medium (*curve b*), and subsequently seem to recover their volume to the same extent (*curve d*) both in the Ca^{2+}-containing (Panel *A*) and in the Ca^{2+}-free medium (Panel *B*). Furthermore, the data appear to be consistent with a uniform RVD response in all cells since the measured data points at $t = 10$ min both in Ca^{2+}-free and Ca^{2+}-containing medium are adequately fitted by the predicted cell volume distribution curve (Panels *C, D, curve d′,* solid line), calculated by simple scaling of curve *b,* assuming a homogeneous RVD response.

In mouse thymocytes Ross and Cahalan (1995) have recently reported that the RVD response is accelerated and enhanced in the subgroup of cells exhibiting swelling-induced $[Ca^{2+}]_i$ transients. To explore whether a similar situation can be demonstrated in the case of Ehrlich cells, the volume distribution curve was recorded 3 min after the hypotonic exposure (*curve c*), at a time when the volume recovery on average is only partial. If the subgroup of cells responding with $[Ca^{2+}]_i$ tran-

Table 2. Effect of BAPTA-loading on the rate of regulatory volume decrease (RVD)

	Rate of regulatory volume decrease (RVD)		
	Δfl/min	Relative to control*	*P*
Control cells	127 ± 21 (7)	1	
Cells loaded with BAPTA in:			
Standard medium	31 ± 1 (3)	0.23 ± 0.01 (2)	<0.01
K^+ equilibrium medium	77 ± 25 (4)	0.84 ± 0.32 (4)	0.3 (n.s.)

* Paired experiments

The cells were loaded with BAPTA as described in Materials and Methods, in either standard medium or K^+ equilibrium medium (at 50 mM K^+), both at pH 7.4. Control cells were handled in parallel (in standard medium) but not exposed to BAPTA-AM. The cells were diluted into hypotonic standard medium (150 mOsm, 50% osmolarity) at zero time, and the cell volume (in fl) followed with time in a Coulter counter. The rate of the RVD response (Δfl/min) was estimated from the linear part of the curve between 1 and 3 min after the hypotonic dilution, using linear regression. For the BAPTA-loaded cells the rate of the RVD response is given relative to control cells (paired experiments). The data are given as the mean ± SEM, with the number of experiments indicated in parentheses. *P* is the level of significance in a paired *t*-test.

sients in the Ca^{2+}-containing medium (6%, *see* Table 1) demonstrate an accelerated and enhanced volume recovery, a heterogeneity should perhaps be detectable in the cell volume distribution curve at this time, in particular since a rise in $[Ca^{2+}]_i$ induced by other means, as discussed below, induces a marked acceleration of the RVD response (*see* Fig. 7). Panel *C* shows that the data points measured in the Ca^{2+}-containing medium at $t = 3$ min (symbols only, same data points as presented in Panel *A*) are adequately fitted by the curve, predicted assuming a homogeneous RVD response with a uniform, partial volume recovery for all cells (*curve c′*, solid line, calculated by simple scaling of curve *b*). This finding, however, would not exclude a heterogeneous response in which a small subgroup (6%) of the cells exhibits an accelerated and enhanced RVD response, since the data points are also adequately fitted by a curve (*curve c″*, broken line), calculated by assuming that 6% of the cells with an initial volume distribution similar to that of the overall cell population exhibit $[Ca^{2+}]_i$ transients and 'complete' volume recovery within the 3-min time period, while the remaining 94% of the cells regulate their volume only corresponding to the shift in modal volume within this time interval. The putative subgroup of cells exhibiting a rapid and 'complete' volume recovery is barely detectable as a small 'shoulder' on the left, rising part of the curve. Model calculations show that such a subgroup of cells only becomes clearly detectable when it amounts to 15–20% or more of the cell population (*not illustrated*). Overall, these data indicate that the majority of cells which do not exhibit $[Ca^{2+}]_i$ transients yet recover their volume adequately, but that the possibility of an accelerated and enhanced RVD response in a small subgroup of cells exhibiting $[Ca^{2+}]_i$ transients cannot be ruled out. It may be noted, though, that such a subgroup of cells exhibiting $[Ca^{2+}]_i$ transients could conceivably be larger for cells attached to the poly-L-lysine-coated coverslips in the microscope chamber than for free floating cells in suspension.

Effect of BAPTA-loading on the RVD Response

Table 2 shows that loading the cells with the Ca^{2+} chelator BAPTA (*see* Materials and Methods), with the aim of buffering $[Ca^{2+}]_i$, almost completely blocks the RVD response. The inhibition is 77% when the cells are loaded with BAPTA in standard medium (pH 7.4), a procedure which reduces $[Ca^{2+}]_i$ from 59 to 26 nM (*see* Jørgensen et al., 1996). Addition of gramicidin (0.5 μM) at the time of maximal cell swelling, to ensure a high K^+ conductance, accelerates the RVD response in Na^+-free hypotonic medium in the BAPTA-loaded cells (*not illustrated*), similar to the findings previously reported in control cells (Hoffmann et al., 1986). BAPTA-loading results in an intracellular acidification (*see* Pedersen et al., 1994) which in itself inhibits the RVD response (Hoffmann et al., 1984). Preincubation of the BAPTA-loaded cells in media at pH_o 8.3 results in an intracellular pH (pH_i) at 7.2, a value close to that ($pH_i = 7.25$) of control cells at pH_o 7.4 (Pedersen et al., 1994; *see* Jørgensen et al., 1996). Under these conditions, the rate of the RVD response in BAPTA-loaded cells (75 ± 11 fl/min, $n = 5$) is still significantly inhibited (about 47%) when compared to control cells (141 ± 1 fl/min, $n = 4$)

at pH_o 7.4 (with pH_i 7.25) (*data not illustrated*). Thus, the inhibitory effect of BAPTA-loading on the RVD response is presumably not only caused by the BAPTA-induced acidification. Loading of Ehrlich cells with BAPTA results, moreover, in a substantial KCl loss and cell shrinkage (*see* Jørgensen et al., 1996) which also in itself presumably would inhibit the RVD response. To counteract this KCl loss, the experiment was repeated with BAPTA-loading in K^+ equilibrium medium (containing 50-mM K^+). Under these conditions, BAPTA-loading of the cells does not significantly reduce the rate of the RVD response, compared to a parallel group of control cells that were not exposed to BAPTA (*see* Table 2).

The Effect of Ca^{2+}-free Media on the Rate of the RVD Response

It has previously been shown that the absence of extracellular Ca^{2+} has no effect on the rate of the RVD response in standard Cl^--containing medium (Hoffmann et al., 1984), also when preceded by preincubation of the cells in Ca^{2+}-free medium with EGTA (Kramhøft et al., 1986). This finding was corroborated in the present study. Monitoring the cell K^+ content following hypotonic exposure in Cl^- medium no difference could be detected between parallel groups at 1-mM Ca^{2+} and at 1-mM EGTA, respectively (*see* Fig. 6, upper panel). A significant reduction (by about 35%) of the rate of the RVD response in Ca^{2+}-depleted cells (preincubated in Ca^{2+}-free medium with 0.5-mM EGTA for 30 min) has, however, been reported for cells in nitrate medium, i.e., under conditions where both cellular and extracellular Cl^- is replaced by nitrate, thereby eliminating any contribution to the RVD response from Cl^--dependent KCl cotransport (*see* Fig. 6 in Kramhøft et al., 1986). Monitoring the RVD response using a Coulter counter after hypotonic exposure in cells preincubated in Ca^{2+}-containing nitrate medium (with 0.1-mM Ca^{2+}) and at zero time transferred to hypotonic nitrate medium, either Ca^{2+}-free (1-mM EGTA) or with 0.1-mM Ca^{2+}, the rate of volume recovery was found to be slightly reduced in acutely Ca^{2+}-free nitrate medium compared to Ca^{2+}-containing medium. In four paired experiments, the rate of volume recovery in the Ca^{2+}-containing medium was 59 ± 4 fl/min, and in the acutely Ca^{2+}-free medium 48 ± 3 fl/min (*not illustrated*). This 18% reduction of the rate of volume recovery in acutely Ca^{2+}-free medium is marginally significant ($P = 0.02$). In similar experiments with preincubation of the cells for 15–30 min in Ca^{2+}-free (2-mM EGTA) and Ca^{2+}-containing (1-mM Ca^{2+}) nitrate medium, respectively, a similar slight, but not significant ($P = 0.13$) inhibition of the rate of the RVD response was found in the Ca^{2+}-free medium, the rate of volume recovery being 69 ± 9 fl/min ($n = 5$) in 1-mM

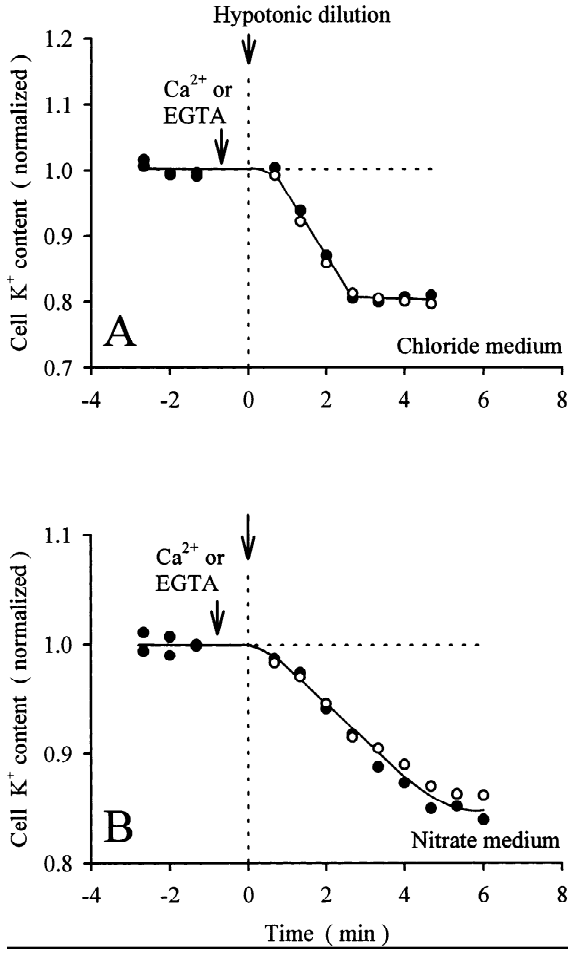

Fig. 6. Volume-induced K^+ loss in the presence and absence of external Ca^{2+}, in Cl^- medium and in nitrate medium. Experimental protocol as in the lower panel of Fig. 1. At zero time parallel groups of the cell suspension were diluted with Ca^{2+}-containing and Ca^{2+}-free hypotonic medium, respectively, such as to reduce the osmolarity by 50%. The cell K^+ content was followed with time (abscissa) and given relative to the initial value (ordinate). All samples nominally represent the same number of cells. All media contained dialyzed albumin (10 mg/ml). *Upper panel:* The cells were suspended in standard medium (Cl^- medium) with 0.1-mM Ca^{2+}. One group received an additional 0.9-mM Ca^{2+} at $t = -0.67$ min (arrow), and was at $t = 0$ (arrow) diluted with hypotonic Cl^- medium containing 1-mM Ca^{2+} (closed circles). The parallel group received 1.1-mM EGTA (with enough NaOH to titrate the protons released by Ca^{2+} chelation) at $t = -0.67$ min (arrow), and was at $t = 0$ (arrow) diluted with hypotonic Cl^- medium containing 0.1-mM Ca^{2+} plus 1.1-mM EGTA (with NaOH) (open circles). Two further experiments with slightly different protocol gave similar results. In these experiments the media were without albumin and contained 1-mM Ca^{2+}, with the Ca^{2+}-free group receiving 2-mM EGTA (with NaOH) at $t = -0.5$ min. *Lower panel:* The cells were preincubated in nitrate medium with 0.1-mM Ca^{2+} for 20–30 min, such as to exchange all cellular and extracellular Cl^- with nitrate. Experimental protocol otherwise as detailed in the upper panel, except that hypotonic nitrate media replaced the Cl^- media. The initial cell K^+ content was similar for the cells in chloride and in nitrate medium. Two further experiments (one with duplicate groups) gave similar results.

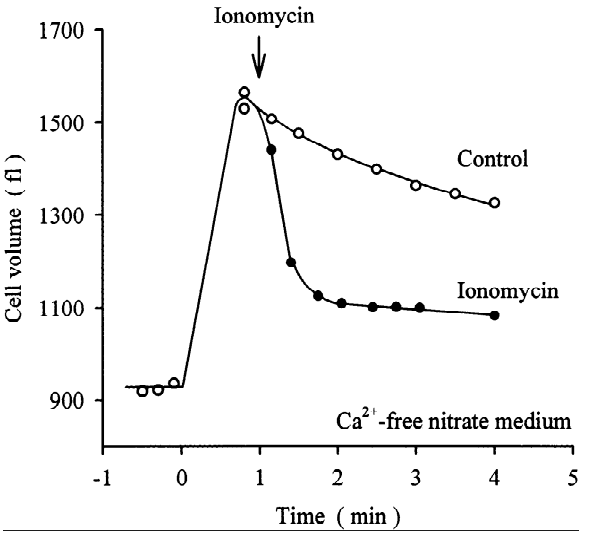

Fig. 7. Regulatory volume decrease in nitrate medium—effect of an increase in $[Ca^{2+}]_i$. The cells were washed once and preincubated for 15–30 min in Ca-free nitrate medium containing 2-mM EGTA, and at zero time diluted 400-fold into hypotonic (150 mM) nitrate medium (Ca^{2+}-free with 2-mM EGTA). The cell volume was followed with time using a Coulter counter. The figure shows the RVD response in the control experiment, and illustrates the effect of addition of the Ca^{2+} ionophore ionomycin (2 μM) 1 min after the hypotonic exposure (arrow), at the time of maximal cell swelling (closed symbols).

Ca^{2+} containing medium, and 51 ± 6 fl/min ($n = 7$) in Ca^{2+}-free medium (2-mM EGTA). Figure 6 (lower panel) shows that when the volume-induced K^+ loss is monitored in nitrate medium in parallel groups in the presence or absence of external Ca^{2+}, no effect upon removal of external Ca^{2+} can be detected, similar to the finding in Cl^- medium (upper panel). Incidentally, in the experiment presented in Fig. 6 the rate of the volume-induced K^+ loss is clearly reduced in nitrate medium compared to the parallel group in Cl^- medium. This is at variance with previous reports that the rate of the RVD response is nearly identical in nitrate and in Cl^- medium in the presence of external Ca^{2+}, and only inhibited in nitrate medium when the medium is Ca^{2+}-free (Hoffmann et al., 1984; Kramhøft et al., 1986). The reasons for this discrepancy are unresolved.

Acceleration of the RVD Response by an Increase in $[Ca^{2+}]_i$

The RVD response can be markedly accelerated by addition of the Ca^{2+} ionophore ionomycin (2 μM) at the time of maximal cell swelling following exposure to hypotonic, Ca^{2+}-free (2-mM EGTA) nitrate medium, as illustrated in Fig. 7. The rate of the RVD response was estimated at 51 ± 6 fl/min ($n = 7$) in control cells and at 565 ± 14 fl/min ($n = 3$) during the first min after addition of ionomycin. The ionomycin-induced release of Ca^{2+} from internal stores and the resulting rise in $[Ca^{2+}]_i$ (*see* Jørgensen et al., 1996) thus accelerates more than 10-fold the loss of KCl and organic osmolytes and of cell water during RVD. A similar acceleration of the RVD response has previously been demonstrated by addition of Ca^{2+} ionophore A23187 in Ca^{2+}-containing Cl^- medium (Hoffmann et al., 1984) and by addition of Ca^{2+}-mobilizing agonists such as thrombin and bradykinin (Simonsen et al., 1990; *see* Hoffmann et al., 1993).

Sensitivity of the Volume-induced K^+ loss to K^+ Channel Blockers

The volume-induced K^+ loss is unaffected by the K^+ channel blocker charybdotoxin (100 mM) and only marginally inhibited by clotrimazole (5 and 10 μM), as seen in Fig. 8*A* and *B*. Charybdotoxin blocks some classes of Ca^{2+}-activated K^+ channels (Miller et al., 1985), and clotrimazole is a potent inhibitor of Ca^{2+}-dependent K^+ channels (Alvarez, Montero & Garcia-Sancho, 1992). That the swelling-induced K^+ loss in Ehrlich cells is insensitive to charybdotoxin confirms an earlier report by Thomas-Young, Smith & Levinson (1993). In the above concentrations charybdotoxin and clotrimazole both strongly inhibit the K^+ loss induced by the Ca^{2+}-mobilizing agonist thrombin in these cells (Harbak & Simonsen, 1995). In contrast, Ba^{2+} (1 mM) inhibits the volume-induced K^+ loss by about 50% (*see* Fig. 8*C*), consistent with previous reports (Lambert, Simonsen & Hoffmann, 1984; *see* Hoffmann et al., 1993). The inhibition by Ba^{2+} was also partial in 5 other experiments with 2-mM Ba^{2+} and in 5 experiments with 5-mM Ba^{2+}, and was only marginally enhanced compared to the inhibition at 1-mM Ba^{2+} (*not illustrated*). Figure 9 shows the dose-dependent inhibition by Ba^{2+} of the volume recovery following hypotonic exposure. The maximal inhibition is about 55%, and $K_{1/2}$ about 0.2 mM. The incomplete inhibition by Ba^{2+} of the RVD response suggests that the K^+ loss during RVD is mediated by two separate K^+ channels, one Ba^{2+}-sensitive and one Ba^{2+}-insensitive. The thrombin-induced K^+ loss in Ehrlich cells is on the other hand almost unaffected by Ba^{2+} (1 mM) (Harbak & Simonsen, 1995). The peptide toxins kaliotoxin (at 100 nM) and margatoxin (at 20 nM), that block the voltage-gated, type *n* (Kv1.3) K^+ channel in lymphocytes (*see* Lewis & Cahalan, 1995), were found not to inhibit the K^+ loss induced by cell swelling (3 experiments, with protocol as in Fig. 8; *not illustrated*). The swelling-induced K^+ loss was also unaffected by TEA (at 1 and 10 mM), a potent blocker of the voltage-gated, type *l* (Kv3.1) channel in lymphocytes (Lewis & Cahalan, 1995) (two experiments with protocol as in Fig. 8; *not illustrated*). Thus, the pharmacological profile of the swelling-induced K^+ loss is entirely different from that of the K^+ loss induced by Ca^{2+}-mobilizing agonists.

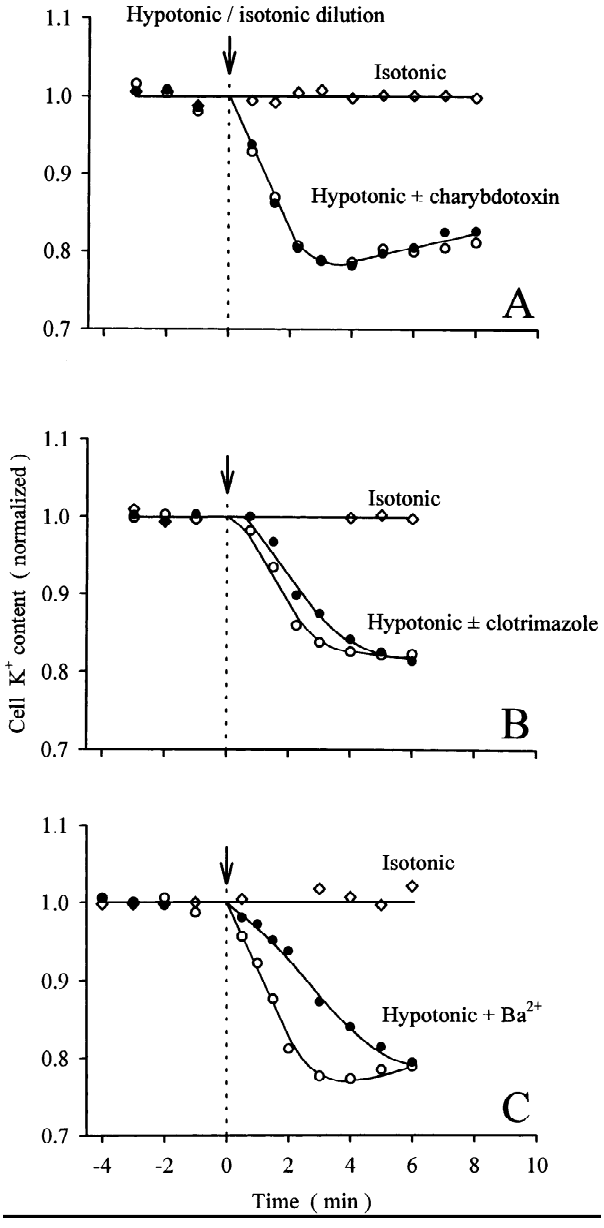

Fig. 8. Effect of K^+ channel blockers on the swelling-induced K^+ loss. Experimental protocol as in Fig. 1 (lower panel). At the time indicated by the arrow parallel groups of the cell suspension were diluted with hypotonic medium, such as to reduce the osmolarity by 50%. The control group was diluted with the same volume of isotonic 1/1 standard medium. The cell K^+ content was followed with time (abscissa) and given relative to the initial value. All media contained dialyzed albumin (10 mg/ml). *A:* One of the experimental groups (closed symbols) received charybdotoxin (100 nM) at $t = -4$ min. One other experiment gave similar results. *B:* One of the experimental groups (closed symbols) received clotrimazole (10 μM) at $t = -4$ min. One other experiment gave similar results. Two further experiments with 5 μM clotrimazole showed, respectively, a similar, marginal inhibition and no detectable inhibition. *C:* One of the experimental groups (closed symbols) received Ba^{2+} (1 mM) at $t = -1$ min. The suspending medium was sulfate-free. One other experiment gave similar results.

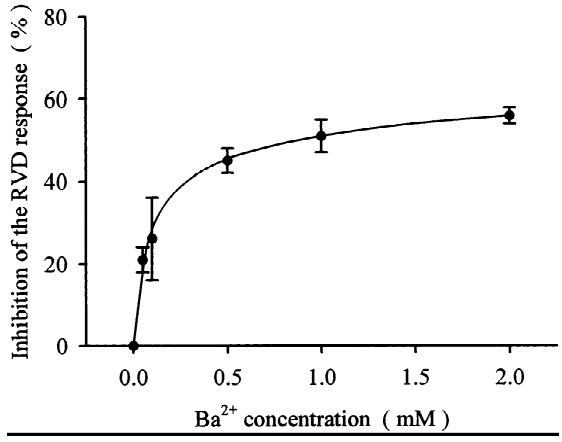

Fig. 9. Inhibition by Ba^{2+} of the volume recovery following hypotonic exposure. The cells were preincubated in isotonic standard medium for 30–60 min before exposure to hypotonic medium (50% osmolarity). The cell volume was monitored in a Coulter counter, and the volume recovery recorded after 5 min, using the maximal cell volume as reference. Ba^{2+} (0.05–2 mM) was added at the time of hypotonic exposure. The Ba^{2+}-induced inhibition of the volume recovery was calculated as $(1 - R_{Ba}/R) \cdot 100\%$ where R and R_{Ba} is the volume recovery in the control and in the presence of Ba^{2+}, respectively. Data are given as the mean of 3 experiments ±SEM, except for 0.05-mM Ba^{2+} which only represents the mean of 2 experiments. The curve was fitted by eye.

Discussion

An increase in $[Ca^{2+}]_i$ has often been assigned a key role in the activation of the RVD response in hypotonic media, and has been proposed to initiate the response by activation of K^+ and Cl^- (and organic osmolyte) channels (*see* Introduction). In many cell types a Ca^{2+} signal has been demonstrated following hypotonic exposure, arising from Ca^{2+} entry and/or Ca^{2+} release from internal stores. In some cell types, though, the role of the Ca^{2+} signal in the activation of the RVD response has been questioned, and in a few cell types no Ca^{2+} signal could be detected following hypotonic exposure (for refs. *see* Introduction).

In the case of the Ehrlich ascites tumor cell, Ca^{2+} has been proposed to play a key role in the activation of the RVD response, and a model has been proposed involving Ca^{2+} entry via stretch-activated Ca^{2+} channels, as well as Ca^{2+} release from internal stores via volume-induced activation of phosphoinositidase C and $InsP_3$ production (*see* Hoffmann et al., 1993). The experimental results have been puzzling, however, and a Ca^{2+} signal during RVD could not be demonstrated in previous studies on the Ehrlich cell (Thomas-Young et al., 1993; Harbak & Simonsen, 1995). These studies would, however, not exclude 'localized' increases in $[Ca^{2+}]_i$ in the cells or asynchronous Ca^{2+}-signaling in the cell population, since the average Ca^{2+}-signal was recorded from cell clusters or from a cell suspension.

The present results show that in the case of the Ehrlich cell a typical RVD response (detected as a decrease in the cell K^+ content) takes place without a concurrent increase in $InsP_3$ (*see* Fig. 1), or in the Ins(1,4,5)P_3 isomer (*see* Results). This finding contradicts earlier reports from this laboratory (Christensen et al., 1988; Svane et al., 1990; Svane & Hoffmann, 1991). The reasons for this discrepancy are unclear. The most likely explanation would appear to be the appearance of extracellular inositol phosphates in the cell suspension, due to damage to a small fraction of the cells by the hypotonic shock, with resulting Ca^{2+}-induced, maximal activation of phosphoinositidase C in the cell debris, and hence with release of Ins(1,4,5)P_3 to the medium in substantial amounts. This interpretation is based on the finding in control cells that the ^{3}H-labeling is 50- to 100-fold higher in PtdIns(4,5)P_2 than in $InsP_3$, and that an about 50-fold increase in $InsP_3$ can be induced by permeabilization of the plasma membrane with digitonin in the presence of 1-mM external Ca^{2+} (Christensen, Harbak & Simonsen, *unpublished experiments*). In earlier experiments, before the introduction of the use of silanized vials and addition of dialyzed albumin to the incubation medium, the $InsP_3$ content in the supernatant was found to account for nearly the total $InsP_3$ content measured in the cell suspension, even under isotonic conditions (Christensen, Harbak & Simonsen, *unpublished experiments*). In the present experiments a significant fraction (about 30%) of the total $InsP_3$ content in the cell suspension was still found in the extracellular compartment (*see* Results). The increase in $InsP_3$ after hypotonic exposure reported in earlier studies (*see above*) was found in experiments performed without the use of silanized vials, without addition of albumin to the media, and without monitoring the inositol phosphate content in the supernatant. Moreover, in those earlier experiments, where the mechanical stimulation presumably was greater, one might also speculate that the release of LTD_4 after hypotonic exposure (Lambert, Hoffmann & Christensen, 1987) was perhaps augmented by the mechanical stimulation, resulting in LTD_4 levels high enough to stimulate leukotriene receptors, with resulting activation of phosphoinositidase C and ensuing $InsP_3$ production in the cells. Stimulation of LTD_4 receptors in Ehrlich cells has been shown to elicit release of $InsP_3$ (Pedersen et al., 1996). Mechanical stimulation such as centrifugation and rapid laminar flow has been reported to stimulate the production of a lipoxygenase product, hepoxilin A_3 in human platelets (Margalit & Livne, 1992). In Ehrlich cells mechanical stimulation can cause ATP release (S. Pedersen, *unpublished experiments*), and this perhaps in our earlier experiments caused receptor-mediated activation of phosphoinositidase C with $InsP_3$ production. Swelling-induced Ins(1,4,5)P_3 production has, apart from preliminary reports on Ehrlich cells (*see above*), also been reported in hepatocytes (Baquet, Meijer & Hue, 1991), rabbit renal proximal tubule cells (Suzuki et al., 1990), and rat liver (Dahl et al., 1991). In the renal tubule cells, however, Suzuki et al. (1990) concluded that Ca^{2+} mobilization played only a minor role in the swelling-induced rise in $[Ca^{2+}]_i$, and in rat liver Dahl et al. (1991) suggested that the observed swelling-induced inositol phosphate response was an epiphenomenon rather than being involved in the activation of volume regulatory K^+ fluxes and in metabolic alterations.

The rapid decrease in PtdInsP_2 following hypotonic exposure reported previously (Christensen et al., 1988) has been confirmed in recent experiments with Ehrlich cells, but this decrease in PtdInsP_2 occurs without concomitant increase in cytosolic inositol phosphates and should not be taken to reflect a swelling-induced activation of phosphoinositidase C, but rather a transient shift to the left in the 'PtdInsP_n-shuttle': PtdIns $\leftrightarrow$ PtdInsP $\leftrightarrow$ PtdInsP_2 (*see* Nielsen et al., 1995; Harbak et al., 1995).

In accord with the lack of change in cellular inositol phosphates, direct measurements of $[Ca^{2+}]_i$ in suspensions of fura-2-loaded cells showed no evidence of Ca^{2+}-signaling after hypotonic exposure (*see* Fig. 2), consistent with earlier reports (Thomas-Young et al., 1993; Harbak & Simonsen, 1995). This absence of Ca^{2+}-signaling during RVD can not be explained by asynchronous Ca^{2+}-signaling in the cell population, since measurements of $[Ca^{2+}]_i$ in single cells likewise demonstrated that no increase in $[Ca^{2+}]_i$ can be detected after hypotonic cell swelling, except in a small subgroup of the cells. Thus, in Ca^{2+} containing media $[Ca^{2+}]_i$ transients are detected in only about 6% of the cells following hypotonic exposure, and reduction (to 100 μM) or removal of Ca^{2+} in the extracellular medium further reduces the number of responding cells (down to about 1%) (*see* Table 1). Moreover, in nonresponding cells no evidence of 'localized' increases in $[Ca^{2+}]_i$ could be detected during RVD, within the spatial resolution of the present technique.

The fura-2-loading of the cells could conceivably buffer $[Ca^{2+}]_i$, and attenuate or block the rise in $[Ca^{2+}]_i$. Such a mechanism, however, seems unlikely to account for the absence of Ca^{2+}-signaling during RVD in the majority of the cells, since Ca^{2+}-mobilizing agonists did elicit Ca^{2+}-signaling (*see* Results). Moreover, it should be noted that the RVD response itself was unaffected by the fura-2-loading (*see* Results).

For those cells that respond (in Ca^{2+}-containing medium) with an increase in $[Ca^{2+}]_i$, the time delay for the response is highly variable—in some cells a rise in $[Ca^{2+}]_i$ is seen with a few seconds after the hypotonic exposure, in others much later, and in about half the cells the increase in $[Ca^{2+}]_i$ is recorded more than 2 min after the hypotonic exposure (*see* Figs. 3 and 4). In particular, the occurrence of the very late responses is not consistent

with the model previously proposed for the activation of the RVD response, according to which a rise in $[Ca^{2+}]_i$ initiates the response (*see* Hoffmann et al., 1993), because the RVD response is initiated already 1/2 to 1 min after the hypotonic exposure and completed within few minutes (*see* Hoffmann et al., 1984, 1986).

The possibility, that only the subgroup of cells that, in Ca^{2+}-containing medium, respond to hypotonic exposure with $[Ca^{2+}]_i$ transients regulate their volume, can be disregarded, since analysis of the volume distribution curves during RVD demonstrates volume recovery also in Ca^{2+}-free medium, and moreover provides no evidence of heterogeneity of the RVD response in the cell population in the presence of external Ca^{2+} (*see* Fig. 5). Moreover, a subgroup of responding cells at 6% (in Ca^{2+}-containing medium) could not account for the swelling-induced K^+ loss that amounts to about 20% of the total K^+ content in the cell suspension (*see* Figs. 1, 6, and 7), nor account for the swelling-induced Cl^- loss, that amounts to about 40% of the total cell content (*not illustrated*). In the small subgroup of cells exhibiting $[Ca^{2+}]_i$ transients the RVD response is presumably accelerated and enhanced, similar to the findings recently reported in the case of mouse thymocytes (Ross & Cahalan, 1995). There is, however, no direct evidence supporting this notion, probably because the subgroup is too small to allow detection in the present analysis (*see* Fig. 5).

In the subgroup of cells in which a swelling-induced rise in $[Ca^{2+}]_i$ was detected, the observed increase in $[Ca^{2+}]_i$ could well be due to Ca^{2+} entry via the stretch-activated, nonselective cation channels that have been demonstrated in Ehrlich cells (*see* Christensen & Hoffmann, 1992). If so, this would explain that such increases in $[Ca^{2+}]_i$ after cell swelling are virtually absent in media with a low Ca^{2+} concentration and in Ca^{2+}-free media (*see* Table 1). The channel activation elicited by membrane stretch during the hypotonic exposure could perhaps be enhanced by adhesive interactions between the attached cells and the poly-L-lysine-coated coverslip in the microscope chamber. Alternatively, as discussed above, release of LTD_4 after hypotonic treatment (*see* Lambert et al., 1987; Lambert, 1994) could perhaps, in particular in Ca^{2+}-containing media, stimulate Ca^{2+}-mobilizing leukotriene receptors in a few cells, leading to a transient increase in $[Ca^{2+}]_i$ (*see* Jørgensen et al., 1996). A similar role could perhaps be played by ATP which can be released from Ehrlich cells by mechanical stimulation (S. Pedersen, *unpublished experiments*).

In experiments with other cell types, a much larger subgroup of cells responding to hypotonic exposure with $[Ca^{2+}]_i$ transients has been reported: 25% and 85% of cultured vascular endothelial cells at 28% and 40% hypotonicity, respectively (*see* Szücs et al., 1996), and about 80% of immature mouse thymocytes at 40% hypotonicity (Ross & Cahalan, 1995). In the latter cell type the swelling-induced $[Ca^{2+}]_i$ transients were dependent upon Ca^{2+} influx.

BAPTA-loading of Ehrlich cells, with the aim of buffering cellular Ca^{2+}, inhibits the RVD response by about 77% (*see* Table 2). This inhibition is presumably to a large extent accounted for by the substantial KCl loss and cell shrinkage caused by the BAPTA-loading (*see* Jørgensen et al., 1996), since it becomes insignificant when the cells are loaded with BAPTA in K^+ equilibrium medium in order to counteract the BAPTA-induced KCl loss (*see* Table 2).

The RVD response has previously been shown to be unaffected by the presence or absence of external Ca^{2+} in standard medium (Hoffmann et al., 1984; Kramhøft et al., 1986). This finding is corroborated in the present study (*see* Fig. 6, upper panel) and argues against a role for Ca^{2+} entry in the RVD response. In the Ca^{2+}-free medium it was conceivable, though, that the volume-induced KCl loss via K^+ and Cl^- channels was perhaps inhibited, but that the inhibition was masked by activation of the putative KCl cotransport system, which has been reported to contribute significantly to the volume-induced KCl loss in Ca^{2+}-free medium for cells preincubated with EGTA (*see* Kramhøft et al., 1986). Any contribution from Cl^--dependent KCl cotransport is eliminated, however, for cells pre-equilibrated in Cl^--free nitrate medium, and under these conditions the rate of the RVD response is also unaffected by the absence of external Ca^{2+} (*see* Fig. 6, lower panel), or at most marginally reduced (*see* Results). These findings thus corroborate that Ca^{2+} entry is playing no role during RVD in Ehrlich cells. In this context it should be noted that one would have expected a marked effect on the RVD response from any hypothetical Ca^{2+} entry associated with RVD, since an increase in $[Ca^{2+}]_i$ induced by other means, as discussed below, dramatically accelerates the rate of the RVD response.

In view of these results, the previously reported acceleration of the RVD response in Ehrlich cells in Ca^{2+}-free medium by addition of 1-mM Ca^{2+} (Hoffmann et al., 1984) is puzzling. It may be noted, though, that the experiment referred to was performed with cells that were Ca^{2+}-depleted following preincubation (15 min) in Ca^{2+}-free medium with excess EGTA, perhaps resulting in depletion-activated Ca^{2+} influx and hence producing a rise in $[Ca^{2+}]_i$ upon restoration of the external Ca^{2+} concentration. In Ehrlich cells a rapid $^{45}Ca^{2+}$ uptake and a rise in $[Ca^{2+}]_i$ upon addition of 1-mM Ca^{2+} to Ca^{2+}-depleted cells has been directly demonstrated (Montero, Alvarez & Garcia-Sancho, 1990).

The finding discussed above that the RVD response is activated without Ca^{2+}-signaling at least in the majority of cells, suggests that the K^+ channels activated by cell swelling and by an increase in $[Ca^{2+}]_i$, respectively,

are separate and distinct. This notion is corroborated by the experiments showing a different sensitivity to K^+ channel blockers for the K^+ channels activated by cell swelling and for those activated by an increase in $[Ca^{2+}]_i$: Whereas the K^+ channels activated by Ca^{2+}-mobilizing agonists in Ehrlich cells are strongly inhibited by charybdotoxin and clotrimazole, but almost insensitive to Ba^{2+} (Harbak & Simonsen, 1995), the volume-induced K^+ channels are insensitive to charybdotoxin and clotrimazole, but inhibited by Ba^{2+} (*see* Fig. 8). In rat thymocytes the RVD response is also insensitive to charybdotoxin (Grinstein & Smith, 1990). In human peripheral blood lymphocytes the RVD response is inhibited by charybdotoxin, but only partially, and charybdotoxin-insensitive K^+ channels account for about 25% of the RVD response (Grinstein & Smith, 1990). Both charybdotoxin-sensitive and -insensitive K^+ channels have been identified and characterized in human T lymphocytes (Lee, Levy & Deutsch, 1992).

The inhibition by Ba^{2+} of the volume-induced K^+ loss and of the RVD response in Ehrlich cells is incomplete (about 50%; *see* Figs. 8 and 9), suggesting the involvement of both Ba^{2+}-sensitive and Ba^{2+}-insensitive K^+ channels in the RVD response. Both of these putative swelling-induced K^+ channels are probably sensitive to quinine, which blocks the RVD response (Hoffmann et al., 1984, 1986). It seems likely that the Ba^{2+}-sensitive K^+ conductance represents the basal K^+ conductance which has been reported to be Ba^{2+}-sensitive (Lang et al., 1987) and to account for about half the total K^+ conductance during the RVD response (Lambert, Hoffmann & Jørgensen, 1989). An alternative interpretation of the difference in Ba^{2+} sensitivity between the K^+ channels activated by Ca^{2+}-mobilizing agonists and by cell swelling could be that Ba^{2+} was inhibiting the K^+ channels from the cytoplasmic side, and that Ba^{2+} entry was occurring during RVD only, via stretch-activated, nonselective cation channels, which were found to conduct Ba^{2+} (Christensen & Hoffmann, 1992). The swelling-induced K^+ channels are insensitive to kaliotoxin and margatoxin (*see* Results), K^+ channel blockers that block the voltage-gated, type *n* (Kv1.3) K^+ channels involved in the RVD response in lymphocytes (*see* Lewis & Cahalan, 1995). The swelling-induced K^+ channels are also insensitive to TEA (*see* Results), a potent blocker of the voltage-gated, type *l* (Kv3.1) K^+ channel in lymphocytes (*see* Lewis & Cahalan, 1995).

Incidentally, the observation that the swelling-induced K^+ channel is distinct from the Ca^{2+}-activated K^+ channel, seems to exclude the proposal (Hoffmann et al., 1986) that, in the absence of Ca^{2+}-signaling during RVD in Ehrlich cells, perhaps the activation of the RVD response could be accounted for by swelling-induced modulation of the Ca^{2+}-sensitivity of the Ca^{2+}-activated K^+ channels.

Although the RVD response in hypotonic medium thus takes place without a detectable increase in $[Ca^{2+}]_i$, a rise in $[Ca^{2+}]_i$ induced by other means at the time of maximal cell swelling will dramatically accelerate the rate of the RVD response. This can be demonstrated as a 10-fold increase in the rate of the RVD response induced by addition of the Ca^{2+} ionophore ionomycin in Ca^{2+}-free medium (*see* Fig. 7), supposedly via an increase in $[Ca^{2+}]_i$ by release of Ca^{2+} from internal stores (*see* Jørgensen et al., 1996). A similar acceleration of the RVD response can be induced by addition of ionophore A23187 (Hoffmann et al., 1984), or by addition of Ca^{2+}-mobilizing agonists (*see* Hoffmann et al., 1993). Since the K^+ conductance is rate-limiting during RVD (Hoffmann et al., 1984, 1986), this increase in the rate of the RVD response after a rise in $[Ca^{2+}]_i$ is presumably due to activation of Ca^{2+}-dependent K^+ channels, distinct from and in parallel to the K^+ channels activated by cell swelling. Ca^{2+}-activated K^+ channels in Ehrlich cells have been described in single-channel studies by Christensen & Hoffmann (1992). Thus, the marked acceleration of the RVD response induced by a rise in $[Ca^{2+}]_i$, rather than suggesting the involvement of $[Ca^{2+}]_i$ in the activation of the RVD response, is presumably analogous to the acceleration seen when a parallel K^+ transport pathway is provided by addition of valinomycin or gramicidin (*see* Hoffmann et al., 1984, 1986).

Thus, during hypotonic cell swelling a detectable increase in $[Ca^{2+}]_i$ is seen only in a small subgroup (6%) of the cells, and then with a highly variable time lag (from a few sec to several min). Moreover, in low Ca^{2+}- and in Ca^{2+}-free media a normal RVD response can be recorded, and under these conditions a detectable increase in $[Ca^{2+}]_i$ is absent in virtually all cells. Finally, as discussed above, the volume-induced K^+ loss is completely insensitive to charybdotoxin, a potent blocker of the Ca^{2+}-activated K^+ channels in Ehrlich cells, and hence any increase in $[Ca^{2+}]_i$ that could possibly be associated with the RVD response, would necessarily be below the $[Ca^{2+}]_i$ level required to activate the Ca^{2+}-dependent K^+ channels. These findings, therefore, consistently show that an increase in $[Ca^{2+}]_i$, by Ca^{2+} entry or release, is not a key factor in the activation of the RVD response in Ehrlich cells.

In the case of the human peripheral blood lymphocyte, a similar conclusion regarding the role of $[Ca^{2+}]_i$ in the RVD response has been reached (for a recent review *see* Grinstein & Foskett, 1990 and Ross & Cahalan, 1995). In this cell type it was initially hypothesized that an elevated $[Ca^{2+}]_i$ during RVD led to opening of Ca^{2+}-activated K^+ channels. Osmotic cell swelling, however, is not associated with a significant increase in $[Ca^{2+}]_i$ in mature T lymphocytes (Grinstein & Smith, 1990), although a recent study has reported a small swelling-induced rise in $[Ca^{2+}]_i$ in human peripheral blood lym-

phocytes (Schlichter & Sakellaropoulos, 1994). Moreover, the RVD response in lymphocytes is unaffected by intracellular Ca^{2+} buffering with BAPTA, and only slightly inhibited by Ca^{2+} depletion (Grinstein & Smith, 1990). The accumulated evidence thus argues against a role for an increase in $[Ca^{2+}]_i$ in the RVD response, and suggests the involvement of voltage-gated K^+ channels (the type *n* or K_v 1.3 isoform) in the swelling-induced K^+ conductance rather than Ca^{2+}-activated K^+ channels (*see* also Deutsch & Chen, 1993). The proposed mechanism of activation of the RVD response in the human lymphocyte is then swelling-induced activation of volume-sensitive Cl^- channels leading to depolarization and in turn activation of voltage-gated K^+ channels with resulting KCl efflux and volume loss. In this context it may be noted that Ehrlich cells also depolarize during RVD (Lambert et al., 1989), but that the swelling-induced K^+ channels as discussed above are insensitive to margatoxin and kaliotoxin, reported blockers of voltage-gated type *n* K^+ channels. In contrast to the mature T lymphocytes, swelling-induced, Ca^{2+} influx-dependent $[Ca^{2+}]_i$ transients have recently been demonstrated in immature mouse thymocytes (Ross & Cahalan, 1995).

Although Ca^{2+} signaling thus appears to play no role in the activation of the RVD response in Ehrlich cells, an unperturbed resting $[Ca^{2+}]_i$ level could play a permissive role and be a prerequisite for a normal RVD response, similar to the proposal by Montrose-Rafizadeh & Guggino (1991) and by Szücs et al. (1996) in the case of rabbit medullary thick ascending limb cells and vascular endothelial cells, respectively. In line with such a notion, a weak or moderate inhibition of the RVD response has been reported in Ca^{2+}-depleted cells (Hoffmann et al., 1984). Moreover, the volume-induced activation of K^+ and Cl^- channels is strongly inhibited by calmodulin antagonists, e.g., pimozide, in similarity to the inhibition of the ionophore A23187 plus Ca^{2+}-induced activation of K^+ and Cl^- channels seen with pimozide (Hoffmann et al., 1984, 1986). A putative Ca^{2+}- and calmodulin-sensitive step in the activation mechanism for the RVD response could be the swelling-induced, transient activation of cytosolic phospholipidase A_2 recently reported by Thoroed et al. (1994, 1996).

It should be noted, however, that some observations seem to argue against a permissive role for the resting $[Ca^{2+}]_i$ level in the RVD response in Ehrlich cells: The marginal inhibition, if any, of the volume recovery seen in Ca^{2+}-free nitrate media is not augmented by preincubation of the cells in the Ca^{2+}-free nitrate medium (*see* Results), although the preincubation presumably reduced the resting level of $[Ca^{2+}]_i$ (*see* Montero et al., 1990; Jørgensen et al., 1996). Moreover, the inhibition of the RVD response in cells where $[Ca^{2+}]_i$ is buffered by BAPTA-loading, is not significant when the BAPTA-loading is performed in K^+ equilibrium medium in order to counteract the BAPTA-induced KCl loss (*see* Table 2), although the resting $[Ca^{2+}]_i$ level under those conditions presumably is significantly reduced, to an extent similar to that seen after BAPTA-loading in standard medium (*see* Jørgensen et al., 1996). The role of the resting $[Ca^{2+}]_i$ level in the RVD response in Ehrlich cells is therefore an open question.

In conclusion, in the case of the Ehrlich ascites tumor cell there is, contrary to previous suggestions no evidence supporting a role for activation of phosphoinositidase C or for Ca^{2+} signaling by Ca^{2+} release or Ca^{2+} entry in the activation mechanism for the RVD response in hypotonic media. It should be noted, though, that the occurrence of 'localized' increases in $[Ca^{2+}]_i$ during the RVD response is not entirely excluded in the present study, due to limitations in the spatial resolution. To explore this possibility, future studies with improved spatial resolution will be needed, e.g., Ca^{2+} imaging by confocal laser scanning microscopy. It is an open question whether an unperturbed resting $[Ca^{2+}]_i$ level plays a permissive role for the RVD response.

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