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# Role of $LTD_4$ in the Regulatory Volume Decrease Response in Ehrlich Ascites Tumor Cells

**N.K. Jørgensen, I.H. Lambert, E.K. Hoffmann**
The August Krogh Institute, Biochemical Department, Universitetsparken 13, DK-2100 Copenhagen Ø, Denmark

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**Abstract.** Stimulation with leukotriene $D_4$ ($LTD_4$) (3–100 nM) induces a transient increase in the free intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) in Ehrlich ascites tumor cells. The $LTD_4$-induced increase in $[Ca^{2+}]_i$ is, however, significantly reduced in $Ca^{2+}$-free medium (2 mM EGTA), and under these conditions stimulation with a low $LTD_4$ concentration (3 nM) does not result in any detectable increase in $[Ca^{2+}]_i$. Addition of $LTD_4$ (3–100 nM) moreover accelerates the KCl loss seen during Regulatory Volume Decrease (RVD) in cells suspended in a hypotonic medium. The $LTD_4$-induced (100 nM) acceleration of the RVD response is also seen in $Ca^{2+}$-free medium and also at 3 nM $LTD_4$, indicating that $LTD_4$ can open $K^+$- and $Cl^-$-channels without any detectable increase in $[Ca^{2+}]_i$. Buffering cellular $Ca^{2+}$ with BAPTA almost completely blocks the $LTD_4$-induced (100 nM) acceleration of the RVD response. Thus, the reduced $[Ca^{2+}]_i$ level after BAPTA-loading or buffering of $[Ca^{2+}]_i$ seems to inhibit the $LTD_4$-induced stimulation of the RVD response even though the $LTD_4$-induced cell shrinkage is not necessarily preceded by any detectable increase in $[Ca^{2+}]_i$. The $LTD_4$ receptor antagonist L649,923 (1 μM) completely blocks the $LTD_4$-induced increase in $[Ca^{2+}]_i$ and inhibits the RVD response as well as the $LTD_4$-induced acceleration of the RVD response. When the $LTD_4$ receptor is desensitized by preincubation with 100 nM $LTD_4$, a subsequent RVD response is strongly inhibited. In conclusion, the present study supports the notion that $LTD_4$ plays a role in the activation of the RVD response. $LTD_4$ seems to activate $K^+$ and $Cl^-$ channels via stimulation of a $LTD_4$ receptor with no need for a detectable increase in $[Ca^{2+}]_i$.

**Key words:** $LTD_4$ — $LTD_4$-receptors — Desensitization — $Ca^{2+}$ — $Ca^{2+}$-depletion — BAPTA — Volume regulation

*Correspondence to:* N.K. Jørgensen

## Introduction

It has previously been suggested that leukotriene $D_4$ ($LTD_4$) is an essential component of the signaling pathways controlling the regulatory volume decrease (RVD) response after hypotonic cell swelling in Ehrlich ascites tumor cells (Lambert, Hoffmann & Christensen, 1987, *see* Hoffmann, Simonsen & Lambert, 1993; Lambert, 1994; Hoffmann & Dunham, 1995). This suggestion was based on the observations that the leukotriene synthesis is stimulated during RVD, that inhibition of the leukotriene synthesis prevents the RVD response, and that $LTD_4$ activates $K^+$ and $Cl^-$-channels as well as the ''taurine channel'' (Lambert et al., 1987; Lambert, 1989; Lambert & Hoffmann, 1993; Lambert & Hoffman, 1994). Diener and Scharrer (1993) similarly found that $LTD_4$ is the messenger for activation of the $Cl^-$-channel during RVD in crypt cells from rat colon epithelium and Mastrocola et al. (1993) found that the swelling activated $Cl^-$ efflux in human fibroblasts was inhibited by the 5-lipoxygenase inhibitor ETH 615-139. Furthermore, Thoroed & Fugelli (1994) demonstrated that the $LTD_4$ antagonist L 660711 strongly inhibited the volume-activated taurine channel in various fish erythrocytes. Involvement of another lipoxygenase product, the 12-lipoxygenase product Hepoxilin $A_3$, in the RVD response was demonstrated for human platelets (Margalit et al., 1993*a*,*b*). In human platelets (Margalit et al., 1993*a*,*b*) as well as in Ehrlich cells (Thoroed et al., 1994) it is thus suggested that cell swelling primarily activates a cytosolic phospholipase $A_2$, resulting in an increased release of arachidonic acid and an increased production of a 12-lipoxygenase product in platelets and a 5-lipoxygenase product in Ehrlich cells (*see* Hoffman & Dunham,

1995). This suggestion is supported by findings in endothelial cells from human umbilical vein indicating that mechanosensitivity in these cells could be mediated by activation of $PLA_2$ and increased availability of arachidonic acid (Oike, Droogmans & Nilius, 1994) as well as by the study of large unilamellar vesicles in which osmotic swelling was found to activate phospholipase $A_2$ directly (Lehtonen & Kinnunen, 1995).

$LTD_4$ reacts with specific G-protein-coupled receptors in several cell types e.g., epithelial cells like an intestinal cell line (Sjölander et al., 1990) and lung tissue (Watanabe et al., 1990) as well as human monocytic leukemia (THP-1) cells (Rochette, Nicholson & Metters, 1993) (*see* Sjölander & Grönroos, 1994 for review). Cells stimulated with $LTD_4$ have been shown to respond with a transient increase in the free intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) in several cell types as e.g., the human THP-1 cells (Chan et al., 1994), the Ehrlich cells (*see* Lambert, 1994), HL-60 cells (Baud, Goetzl & Koo, 1987) and an intestine epithelial cell line (Sjölander et al., 1990) (*see* Sjölander & Grönroos, 1994 for review). It has been suggested that $LTD_4$ receptors via a G protein could activate the phospholipase C signaling pathway resulting in inositol(1,4,5)$P_3$ and $Ca^{2+}$ mobilization (*see* Sjölander & Grönroos, 1994).

Desensitization of the $LTD_4$ receptor after 1 min of previous stimulation with $LTD_4$ was observed e.g., in THP-1 cells (Chan et al., 1994), in HL-60 cells (Baud et al., 1987) and after 7.5 min of pretreatment in rat basophilic leukemia cells (Winkler, Mong & Crooke, 1988). This means that after stimulation with $LTD_4$ for 1 and 7.5 min, respectively, it was not possible to provoke an increase in $[Ca^{2+}]_i$ by a second stimulation with $LTD_4$.

According to Winkler et al. (1988) a second response was not detected even 60 min after the first stimulation. The desensitization was homologous, since pretreatment with $LTD_4$ had no effect on the increase in $[Ca^{2+}]_i$ seen after stimulation with other $Ca^{2+}$ mobilizing agonists ($LTB_4$, thrombin, ATP).

In the present report, we confirm preliminary results by Lambert (1994) that $LTD_4$ produces a transient increase in $[Ca^{2+}]_i$ in Ehrlich cells. The major goals of this study are (*i*) to elucidate whether or not the $LTD_4$-mediated activation of $K^+$ and $Cl^-$ channels is a direct effect of $LTD_4$ or secondary to the $LTD_4$-induced increase in $[Ca^{2+}]_i$, (*ii*) to investigate whether the $LTD_4$-induced channel activation shows desensitization to $LTD_4$ in similarity to the $LTD_4$-induced $Ca^{2+}$-signaling and (*iii*) to see whether the RVD response is inhibited when the cells have been prestimulated with $LTD_4$.

Part of this investigation has previously been published at the Scandinavian Physiological Society meeting in Göteborg 1994 in an abstract form (Jørgensen, Lambert & Hoffmann, 1994).

## Materials and Methods

### Cell Suspension

Ehrlich ascites tumor cells (hyperdiploid strain) were maintained and harvested as described previously (*see* Hoffmann, Lambert & Simonsen, 1986). The washed cells were suspended at 4% cytocrit in standard medium, and incubated for about 30 min before the experiments. Loading of the cells with fura-2, BCECF or BAPTA (*see below*) was initiated during this period. All experiments were conducted at 37°C. In experiments in which the cells were suspended in nitrate medium, $Ca^{2+}$-free medium, low $Ca^{2+}$ medium, or medium with pH 8.3, an additional wash of cells in the experimental medium was performed after 15 min preincubation.

### Incubation Media

(*A*) Standard medium (300 mOsm) contained (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 (*B*) $NaNO_3$ medium was prepared by substituting the $Na^+$ and the $K^+$ salts of $NO_3^-$ for NaCl and KCl. (*C*) low $Na^+$ medium (NMDG medium, 2 mM $Na^+$) was prepared by substituting 148 mM N-methyl-D-glucammonium for 148 mM $Na^+$ (*D*) $Ca^{2+}$-free medium was prepared using 2 mM EGTA as a $Ca^{2+}$-buffer and omitting $CaCl_2$. (*E*) Hypotonic medium (150 mOsm) was prepared by a 1:1 dilution of the standard medium with distilled water containing buffers in concentrations as in the standard medium. The concentrations of $Ca^{2+}$ in the hypotonic media were kept at 1 mM or 0 mM using EGTA as a $Ca^{2+}$ buffer. (*F*) Media with pH 8.3 were prepared by replacing MOPS, TES and HEPES with 5 mM TRICINE and 5 mM BICINE.

### Reagents and Radioisotopes

All reagents were of analytical grade and obtained from Sigma, unless otherwise indicated. Fura-2-AM, Fura-2-P (pentapotassium salt), BCECF and BAPTA-AM were obtained from Molecular Probes (OR). Bumetanide was a gift from Leo Pharmaceuticals (Ballerup, Denmark). Ionomycin, digitonin, valinomycin, gramicidin D, A23187, arachidonic acid, bradykinin, thrombin and poly-L-lysine were obtained from Sigma. $LTD_4$ was obtained from Cascade Biochem Ltd. (Berkshire, UK). L649,923 was obtained from Merck Frosst Canada. Silicone oils AR20 and AR200 were from Wacker Chemie (Vienna, Austria). Chelerythrine was obtained from Alamone Labs (Jerusalem, Israel). Pimozide was obtained from Jannsen Biochemica. $^3$H-inulin and $^{36}$Cl were obtained from Amersham International plc, England, whereas $^{86}$Rb was obtained from Risø, Denmark. *Stock solutions* (*agonists*): $LTD_4$ was added from a 100 μM stock solution in ethanol. Bradykinin was added from a 1 mM stock solution in distilled water. Thrombin was added from a 1,000 I.U./ml stock solution in distilled water.

### Measurements of Cell Volume

Cell volume was estimated as the water content (ml/g dry wt) as described in Hoffmann et al. (1983) or by electronic cell sizing as described in Hoffmann, Simonsen & Lambert (1984) using a Coulter counter model ZB equipped with a Coulter channellyzer (C-1000) and a cell suspension with a final cell density of approximately 90,000 cells per ml, which is equivalent to a cytocrit of about 0.008%.

## $^{36}Cl^-$ Efflux, $^{86}Rb^+$ Efflux and Rate Constants

Ehrlich cells, equilibrated with $^{36}Cl^-$ ($1.7 \cdot 10^4$ Bq/ml) in standard medium for 30 to 40 min at cytocrit 0.8% (control cells) or 0.4% (for cells to be loaded with BAPTA), were packed by centrifugation, washed once in standard medium, and then resuspended in the experimental medium. The final experimental cytocrit was 4%. The cellular $^{36}Cl^-$ activity was estimated by transferring 0.5 ml cell suspension to preweighed vials and separating the cells from the medium by centrifugation (20,000 × *g*, 60 sec) 50 μl of the supernatants were diluted 10 times with 70% perchloric acid (7% final concentration) and saved for determination of extracellular activity. Excess supernatant was removed by suction and the wet weight of the cell pellet was determined by reweighing the samples. The packed cells were then lysed in 400 μl distilled water, deproteinized by addition of 50 μl 70% perchloric acid and centrifuged (20,000 × *g*, 10 min). The supernatant was used for determination of cellular $^{36}Cl^-$ activity and the perchloric acid precipitate was dried (90°C, 48 h) and used for determination of the cell dry weight (*see* Lambert, Hoffmann & Jørgensen, 1989). Cellular $^{36}Cl^-$ activity (cpm/g cell dry weight) was corrected for $^{36}Cl^-$ activity trapped in the extracellular medium using $^3H$-inulin as marker (Hoffmann, Simonsen & Sjøholm, 1979). Ehrlich cells, equilibrated with $^{86}Rb^+$ ($10^4$ Bq/ml) in standard medium for 30 min at cytocrit 0.8% (control cells) or 0.4% (cells to be loaded with BAPTA) were packed and washed in standard medium and then resuspended in the experimental solution. The final experimental cytocrit was 4%. The $^{86}Rb^+$ efflux was followed with time by serially isolating cell-free efflux medium by centrifugation of 500 μl cell suspension through a silicone oil phase (300 μl: 1 part 20 AR/1 part AR 200). The extracellular $^{86}Rb^+$ activity (cpm/ml medium) was estimated in 100 μl of the supernatant and converted to cpm/g dry wt by division with the dry weight (g/ml medium).

$^3H^+$, $^{36}Cl^-$ and $^{86}Rb^+$ activity were measured in a liquid scintillation spectrometer (Packard TRI-CARB 460C Liquid Scintillation System) using ULTIMA GOLD™ (Packard) as scintillation fluid.

The rate constant ($k$) for the unidirectional $^{36}Cl^-$ efflux and $^{86}Rb^+$ efflux were calculated from the equations:

$$a_t = a_\infty + (a_o - a_\infty) \cdot e^{-kt} \quad (1)$$

$$a_t = a_o + (a_\infty - a_o) \cdot (1 - e^{-kt}) \quad (2)$$

respectively where $a_t$, $a_o$ and $a_\infty$ are the cellular activities (cpm/g cell dry wt) at time $t$, at zero time and at isotope equilibrium, respectively (*see* Hoffmann et al., 1979). The unidirectional flux of $Cl^-$ ($J_{Cl}$) and $K^+$ ($J_K$) were calculated as the product of the rate constants, $k$ ($min^{-1}$) and the cellular $Cl^-$ or $K^+$ content (μmol/g cell dry wt), respectively.

## Measurements of $Na^+$, $K^+$ and $Cl^-$ Content

$Na^+$ and $K^+$ were determined by atomic absorption flame photometry and $Cl^-$ was assessed by coulometric titration as described in Lambert et al., 1989.

## Loading of Ehrlich Ascites Tumor Cells with fura-2-AM and BAPTA-AM

Ehrlich cells (cytocrit 0.4%) were incubated with 2 μM fura-2-AM or 50 μM BAPTA-AM in standard medium with 0.2% (w/v) bovine serum albumin (BSA) for 20 min (fura-2 loading) or 35 min (BAPTA loading) at 37°C, washed once with fresh buffer containing 0.2% BSA in order to remove extracellular fura-2 or BAPTA and once with the experimental medium.

## Measurements of the Free Intracellular $Ca^{2+}$ Concentration in *Single Cells*

Fluorescence was recorded with a Zeiss Axiovert 10 fluorescence microscope equipped with a 40×/1.30 NA oil immersion achrostigmat (UV) objective. The fura-2 loaded cells were diluted to cytocrit 0.3% and placed in a thermostatically controlled (37°C) chamber (POC, Biophysica Technologies), the coverslips were coated with poly-L-lysine (25 mg/ml) in order to improve cell attachment. The cells were excited by a 75 W Xenon lamp using a K12 filter and a BPB 380/20 filter in the excitation light path to protect the cells against infrared illumination and to adjust the intensity of the excitation light. Dual excitation wavelengths of 340 nm and 380 nm were obtained by BP 340/10 and BP 380/10 filters. Filters were placed in an automated filter wheel (LUDL Electronic Products) and selected by computer under control of the digital image processing and quantitative fluorescence system (Image/Fluor, Universal Imaging Corporation). A shutter was used to control illumination. Emitted light was passed through a BSP 425 dichroic mirror and filtered by a BP 500–530 filter. The fluorescence was viewed by an intensified CCD camera (CCD72 with a GenIIsys intensifier from Date-MTI). The camera response was linear over the measured range of fluorescence intensities. The images were collected as an average of 6 frames after 340 nm and 380 nm excitation, respectively and the ratio of the images obtained after 340 nm over the images obtained after 380 nm excitation was calculated on a pixel-to-pixel basis after background subtraction. Cells loaded with fura-2 displayed bright stable fluorescence, whereas unloaded cells possessed no detectable autofluorescence at the camera and intensifier gain employed.

The cells were stimulated by (i) addition of 10 μl stock solution (*see* Reagents for concentrations) of the agonist directly to the cells using a pipette or by (ii) addition of a large volume (typically 2 ml to the experimental chamber containing 2-ml cell suspension) of a solution containing the agonist or of a hypotonic medium.

The maximal change in the ratio (ΔR) was calculated by subtraction of the average ratio in the unstimulated cell from the maximal ratio obtained after stimulation. The maximal change in $[Ca^{2+}]_i$ was calculated by subtraction of the average $[Ca^{2+}]_i$ in the unstimulated cell from the maximal $[Ca^{2+}]_i$ measured after stimulation.

## Measurement of the Free Intracellular $Ca^{2+}$ Concentration in *Cell Suspensions*

Cells loaded with fura-2 were resuspended at a cytocrit of 5% in the medium used for the experiment. The fura-2 fluorescence measurements were performed on 3 ml suspension with a 0.5% cytocrit obtained by a second dilution of the cell suspension into the experimental medium. Fluorometric measurements were performed in polystyrene cuvettes (Elkay Ultra-VU) in a Perkin Elmer LS-5 Luminescence Spectrometer using excitation wavelengths of 340 nm and 380 nm and measuring the emission at 510 nm. The excitation and emission slit widths were 5 nm. The temperature of the cuvette was thermostatically controlled to 37°C and the cell suspension was continuously stirred by use of a Teflon-coated magnet, driven by a motor attached to the cuvette house.

At the beginning and at the end of the experiment a sample of the cell suspension (same cytocrit as in the experiments) was centrifuged and fluorescence of the extracellular medium was measured. These values were used for background correction. Background intensities

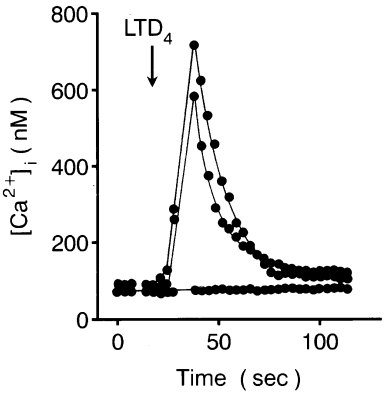


**Fig. 1.** Stimulation with $LTD_4$ results in a transient increase in $[Ca^{2+}]_i$. Ehrlich cells, loaded with fura-2 as described in Materials and Methods, were suspended in standard incubation medium containing 1 mM $Ca^{2+}$ and preincubated for 10–50 min. Final experimental cytocrit was 0.2%. $[Ca^{2+}]_i$ was measured in *single cells* using fluorescence microscopy. The $[Ca^{2+}]_i$ values were calculated from the fluorescence signal at 340 nm excitation divided by the fluorescence signal at 380 nm excitation (emission constantly measured at 510 nm) as described in Materials and Methods. The figure shows $[Ca^{2+}]_i$ measured in three individual cells as a function of time. $LTD_4$ (100 nM) was added (as a large volume, *see* Materials and Methods) as indicated by the arrow. The data are representative of seven experiments.

were subtracted from the measured fluorescence intensities before calculation of the 340 nm/380 nm ratio. The autofluorescence from unloaded cells was negligible.

The maximal change in the ratio ($\Delta R$) and the maximal change in $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$) were calculated as described above.

## Measurements of Intracellular pH

Intracellular pH was measured as described by Pedersen et al. (1996).

## In vitro Calibration of the Fura-2 Fluorescence Signal

### *Single Cell Experiments*

The fluorescence signal from 10 μl of calibration solution between two coverglasses was recorded. Calibration solutions consisted of 10 μM fura-2 pentapotassium salt (fura-2-P) in (mM): 158 $K^+$, 158 $Cl^-$, 1 $Mg^{2+}$, 1 $SO_4^{2-}$, 1 $HPO_4^{2-}$, 3.3 MOPS, 3.3 TES and 5 HEPES at pH 7.40 with a free $Ca^{2+}$ concentration adjusted to 0 or 1 mM using EGTA (*see* incubation Media). The free $Ca^{2+}$ concentration was calculated from the measured ratio values according to the equation:

$$[Ca^{2+}] = K_d \cdot ((R - R_{min})/(R_{max} - R)) \cdot S_{f380}/S_{b380} \quad (3)$$

where $K_d$ is the dissociation constant (224 nM, *see* Grynkiewicz, Poenie & Tsien, 1985) and $R$ is the fluorescence ratio at 340 nm and 380 nm excitation. $R_{max}$ and $R_{min}$ are the equivalent fluorescence ratios of fura-2 at saturating $Ca^{2+}$ concentrations, and in $Ca^{2+}$-free medium (with 2 mM EGTA), respectively. $S_{f380}$ and $S_{b380}$ are proportionality coefficients, measured from the fluorescence intensity at 380 nm excitation using calibration solutions containing zero or saturating $Ca^{2+}$ concentrations, respectively (Grynkiewicz et al., 1985). The in vitro values for $R_{max}$, $R_{min}$ and $S_{f380}/S_{b380}$ were estimated at 9.1, 0.3 and 7.2, respectively. As described below, the excitation spectra obtained from the fura-2 loaded cells were identical to the spectra measured using fura-2-P, indicating that in vitro calibration could be used.

### *Cell Suspension Measurements*

$R_{max}$, $R_{min}$ and $S_{f380}/S_{b380}$ were determined using 3 ml of calibration solution (described above, except that the concentration of Fura-2-P was 0.5 μM) in a cuvette and the fluorescence intensity at the appropriate excitation wavelength was recorded as described above. The values for $R_{max}$, $R_{min}$ and $S_{f380}/S_{b380}$ were determined at 20.6, 0.8 and 9.3, respectively. The free $Ca^{2+}$ concentration was calculated from the corresponding ratio values as described above for *single cells.*

The average resting level of $[Ca^{2+}]_i$ was in cell suspensions estimated at 160 ± 6 nM ($n$ = 73) and in single cells at 59 ± 2nM ($n$ = 271) (standard medium, 1 mM $Ca^{2+}$). The higher value found in cell suspensions reflects the difference between the methods. In cell suspension measurements, the signal reflects the response from the total cell population, including damaged cells and debris whereas in single cell experiments only viable cells are recorded.

The excitation spectra of fura-2-P in calibration solutions with zero or saturating $Ca^{2+}$ concentrations were measured and compared to excitation spectra measured on a fura-2 solution obtained from fura-2-AM loaded cells. This fura-2 solution was prepared by treating the fura-2-AM loaded cell suspension (in media with zero or saturating $Ca^{2+}$ concentrations, respectively) with digitonin (0.5 mg/ml) followed by centrifugation. The excitation spectra of the supernatant was then measured. These spectra were similar to those obtained for fura-2-P and thus indicated that in vitro calibration could be used.

## Statistical Evaluation

Values are given as the mean ± SEM, with the number of experiments ($n$) indicated in brackets. Students $t$-test was used to evaluate statistical significance.

## Abbreviations

DMSO: dimethylsulfoxide; EGTA: ethylene-glycol-bis-(β-aminoethyl-ether)N,N,N′,N′-tetraacetic acid; BSA: Bovine serum albumin; AM: acetoxymethyl ester; BAPTA: 1,2-bis(*o*-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid; NMDG: N-methyl-D-glucammonium; TRICINE: N-tris(hydroxymethyl)methyl-glycine; BICINE: N,N-bis(2-hydroxyethyl)-glycine; MOPS: 3-(N-morpholino) propane sulfonic acid; TES: N-tris-(hydroxymethyl)methyl-2-aminoethane sulfonic acid; HEPES: N-2-hydroxyethylpiperazine-N′2-ethanesulfonic acid; $LTD_4$: Leukotriene $D_4$, BCECF: 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein.

# Results

## The effect of $LTD_4$ on the Free Intracellular $Ca^{2+}$ Concentration

Stimulation of Ehrlich ascites tumor cells with $LTD_4$ results in a fast increase in $[Ca^{2+}]_i$ followed by a down-

**Table 1.** Quantification of the increases in $[Ca^{2+}]_i$ and the acceleration of the rate of the RVD response after addition of $LTD_4$

| $LTD_4$ added nM | Standard medium 1 mM $Ca^{2+}$ $\Delta[Ca^{2+}]_i$ nM | Standard medium $Ca^{2+}$-free, 2 mM EGTA $\Delta[Ca^{2+}]_i$ nM | Hypotonic medium $Ca^{2+}$-free, 2 mM EGTA RVD $\Delta$ fl/min |
|---|---|---|---|
| 100 | 290 ± 31 (12) | 80 ± 19 (6) | — |
| 10 | 243 ± 82 (6) | 19 ± 4 (9) | 392 ± 20 (3) |
| 3 | 207 ± 81 (5) | 5 ± 2 (7) | 295 ± 21 (4)[a] |
| 0 | — | — | 140 ± 12 (4)[b] |

The fura-2 loaded Ehrlich cells were suspended in isotonic standard medium with 1 mM $Ca^{2+}$ or in $Ca^{2+}$-free standard medium with 2 mM EGTA and preincubated for 10–50 min. Measurements of $[Ca^{2+}]_i$ were obtained from a *cell suspension* (final experimental cytocrit 0.5%) using a fluorescence spectrophotometer (*see* Materials and Methods). The change in the $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$) was calculated as described in Materials and Methods. Measurements of cell volume were obtained from a cell suspension using a Coulter counter (*see* Materials and Methods). At zero time the cells were exposed to hypotonicity (*see* Fig. 2*B*). $LTD_4$ (3 nM or 10 nM) was added 1 min after the reduction in osmolarity. The rate of the regulatory volume decrease ($\Delta$fl/min) was calculated as the volume recovery within the first min after addition of $LTD_4$ using linear regression.
[a] Addition of 3 nM $LTD_4$ to cells suspended in hypotonic $Ca^{2+}$-free medium (1 mM EGTA) also did not result in any detectable increase in $[Ca^{2+}]_i$ (*see* Fig. 2*A*).
[b] Cells hypotonically diluted in $Ca^{2+}$ free medium show no detectable increase in $[Ca^{2+}]_i$ (Jørgensen et al., 1996).

regulation towards the resting level of $[Ca^{2+}]_i$. This is seen in Fig. 1 which demonstrates the change in $[Ca^{2+}]_i$ as a function of time in the presence of 1 mM extracellular $Ca^{2+}$ after addition of $LTD_4$ (100 nM) as measured in *single cells* using fluorescence microscopy and the fluorescent probe fura-2. Similar data have been obtained in preliminary experiments with $LTD_4$ (100 nM) using a *suspension of cells* and a fluorescence spectrophotometer (*see* Lambert, 1994). As seen from Fig. 1, the cell population is heterogenous with respect to the response to $LTD_4$ and some cells never respond. For the responding cells the mean peak time is 26 ± 3 sec after addition of $LTD_4$ ($n = 25$, isotonic standard medium, 1 mM $Ca^{2+}$). This type of heterogeneity is, however, also seen after stimulation with other agonists, e.g., thrombin or bradykinin (*data not shown*) which are known to result in $Ca^{2+}$ mobilization via an increase in $Ins(1,4,5)P_3$ (Simonsen et al., 1990). Table 1 shows the effect of different concentrations of $LTD_4$ on $[Ca^{2+}]_i$ in *cell suspensions.* The data demonstrate that in $Ca^{2+}$-containing media addition of 3–100 nM $LTD_4$ results in increases in $[Ca^{2+}]_i$, whereas in $Ca^{2+}$-free media (with 2 mM EGTA) 3 nM $LTD_4$ can not provoke any measurable increase in $[Ca^{2+}]_i$, stimulation with 10 nM $LTD_4$ causes only small increases in $[Ca^{2+}]_i$, and even at 100 nM $LTD_4$ the $Ca^{2+}$ response is reduced as compared to the response in $Ca^{2+}$ containing media. Thus, a large part of the $Ca^{2+}$ response is caused by $Ca^{2+}$ influx at low concentrations of $LTD_4$. Maximal stimulation with $LTD_4$ at the *single cell* level using fluorescence microscopy (final $LTD_4$ concentration in the chamber is ~500 nM) is found to increase $[Ca^{2+}]_i$ up to ~990 nM in the presence of 1 mM $Ca^{2+}$ extracellularly and to ~580 nM in $Ca^{2+}$-free media. This indicates that the increase in $[Ca^{2+}]_i$ after stimulation with $LTD_4$ concentrations in the lower nanomolar range predominantly results from influx of $Ca^{2+}$, whereas stimulation with higher concentrations also result in a significant release of $Ca^{2+}$ from internal stores.

## The Effect of $LTD_4$ on the Regulatory Volume Decrease Response

It has previously been shown that $LTD_4$ (60 nM) accelerates the RVD response in hypotonic $Ca^{2+}$ containing media (Lambert et al., 1987; Lambert, 1989). The dose giving half maximal stimulation was estimated at ~15 nM (Lambert, 1989) and at ~8 nM (Lauritzen et al., 1993). The following experiments were performed to examine the effect of low $LTD_4$ concentrations in $Ca^{2+}$-free media. Figure 2*A* demonstrates that, in hypotonic $Ca^{2+}$-free media, stimulation of fura-2 loaded Ehrlich cells with 3 nM $LTD_4$ does not induce a significant increase in $[Ca^{2+}]_i$, whereas addition of a higher concentration of $LTD_4$ (100 nM) as indicated by the second arrow results in a transient increase in $[Ca^{2+}]_i$, thus indicating that the cells are able to respond with a change in $[Ca^{2+}]_i$ after $LTD_4$ stimulation also under hypoosmotic conditions. Figure 2*B* demonstrates, that addition of 3 nM $LTD_4$ to cells suspended in hypotonic $Ca^{2+}$-free medium is still able to accelerate the RVD response significantly. Table 1 summarizes the effect of addition of 3 and 10 nM $LTD_4$ in $Ca^{2+}$-free media on the rate of the RVD response and the $LTD_4$ induced increase in $[Ca^{2+}]_i$ (measured in cell suspensions, *see above*). It is seen, as illustrated in Fig. 2, that stimulation with 3 nM $LTD_4$, although it does not result in any detectable increase in $[Ca^{2+}]_i$, accelerates the RVD response significantly ($P < 0.005$). Thus, acceleration of the RVD response by $LTD_4$ seems not to require any measurable increase in $[Ca^{2+}]_i$. The increase in the rate of the RVD response after addition of 10 nM $LTD_4$ is larger than after addition of 3 nM $LTD_4$, and at

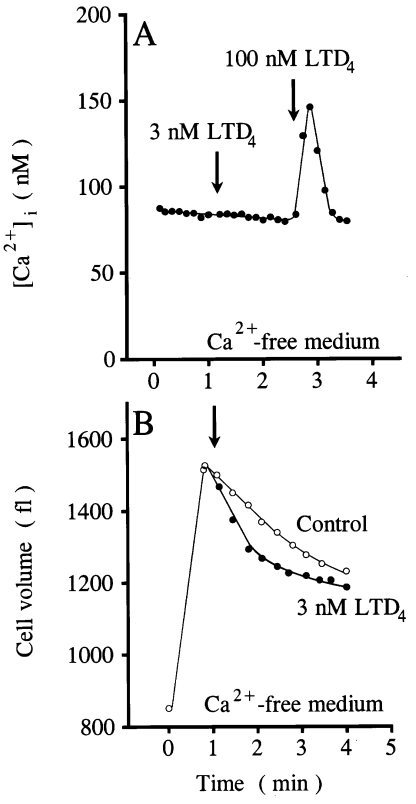


**Fig. 2.** Effect of $LTD_4$ on $[Ca^{2+}]_i$ and the regulatory volume decrease in hypotonic $Ca^{2+}$-free medium. (*A*) Ehrlich cells were loaded with fura-2 and preincubated for 15–30 min in $Ca^{2+}$-free standard medium (300 mOsm) containing 2 mM EGTA and at time zero diluted to 50% of the normal osmolality by addition of buffered water. $[Ca^{2+}]_i$ was followed in the *cell suspension* using a fluorescence spectrophotometer (cytocrit 0.5%). $LTD_4$ (3 nM or 100 nM) was added as indicated by the arrows. It should be noted that calibration values obtained under isotonic in vitro conditions are used to convert the measured 340 nm/380 nm ratio values into $Ca^{2+}$ concentrations. This might introduce a minor error in the estimation of exact values $[Ca^{2+}]_i$ as the decrease in viscosity and ionic strength may influence the $K_d$ for $Ca^{2+}$ binding to fura-2 as well as the fluorescence signal (Grynkiewicz et al., 1985; Roe, Lemasters & Herman, 1990). The data are from a single experiment, representative of 6 experiments, with addition of 3 nM $LTD_4$ in hypotonic $Ca^{2+}$-free medium. (*B*) Ehrlich cells were preincubated at cytocrit 4% in $Ca^{2+}$-free standard medium (300 mOsm) containing 2 mM EGTA for 15–30 min and at time zero diluted 400-fold with hypotonic (150 mOsm) $Ca^{2+}$-free NaCl medium containing 2 mM EGTA. Cell volume (fl) was followed with time using a Coulter counter. The data demonstrate a control response (open symbols) and an experiment with addition of $LTD_4$ (3 nM) at the time of maximal cell swelling as indicated by the arrow (closed symbols). The data are from a representative single experiment out of four experiments.

the higher concentrations there is a small increase in $[Ca^{2+}]_i$. It should be noted, that the $Ca^{2+}$-measurements in Table 1 are performed in isotonic medium. Hypotonically diluted cells in $Ca^{2+}$-free medium show no detectable increase in $[Ca^{2+}]_i$ (Jørgensen et al., 1996). Stimulation with 3 nM $LTD_4$ when added to a cell suspension in $Ca^{2+}$-free hypotonic medium, in agreement with the data obtained in $Ca^{2+}$-free isotonic medium, also does not result in any detectable increase in $[Ca^{2+}]_i$ (*see* Fig. 2*A*).

### Effect of a Leukotriene receptor Antagonist

A selective leukotriene receptor antagonist, L649,923 (Jones et al., 1986), was previously found to inhibit the RVD response as well as the $LTD_4$-induced acceleration of the RVD response (Lambert, 1989). Figure 3 is a dose-response curve for the effect of L649,923 on the $LTD_4$-induced acceleration of the RVD response. It is seen that 1 μM of L649,923 is sufficient to completely block the effect of $LTD_4$ (100 nM) addition. Figure 4*A* and *B* shows the results of experiments performed to investigate the effect of L649,923 on the $LTD_4$-induced increase in $[Ca^{2+}]_i$ (measured in cell suspensions). It is seen that stimulation with 100 nM $LTD_4$ results in a significant transient increase in $[Ca^{2+}]_i$ and that the two agonists bradykinin (10 μM) and thrombin (10 I.U./ml) still result in an increase in $[Ca^{2+}]_i$ when added after $LTD_4$ (Fig. 4*A*). However, the increase in $[Ca^{2+}]_i$ induced by $LTD_4$ is completely blocked by 1 μM L649,923, whereas the increases in $[Ca^{2+}]_i$ induced by bradykinin and thrombin, are unaffected by L649,923 (Fig. 4*B*). This demonstrates that the antagonist L649,923 specifically inhibits the $LTD_4$-induced increase in $[Ca^{2+}]_i$, indicating that $LTD_4$ exerts its effect on $[Ca^{2+}]_i$ via a specific leukotriene receptor. In addition to

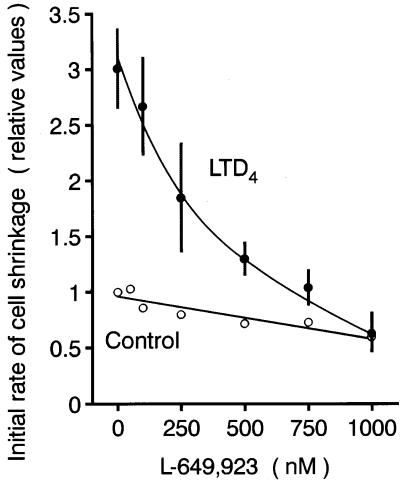


**Fig. 3.** Effect of the $LTD_4$-receptor antagonist, L649,923, on the $LTD_4$-induced acceleration of the RVD response. The cells were treated as described in the legend to Fig. 2*B*, except that the extracellular $Ca^{2+}$ concentration was 0.5 mM. L649,923 (0–1000 nM) was added at the time of hypotonic exposure and $LTD_4$ (100 nM) was added 1 min after the reduction in osmolarity (*see* Fig. 2*B*). The rate of the regulatory volume decrease (Δfl/min) was calculated as the cell shrinkage within the first min after addition of $LTD_4$ using linear regression. Control cells (open symbols) only received L649,923. Values are given relative to the water loss in control cells without addition L649,923 and $LTD_4$. The curve with control cells is the mean of two sets of experiments, whereas the curve with $LTD_4$ is the mean of three sets of experiments.

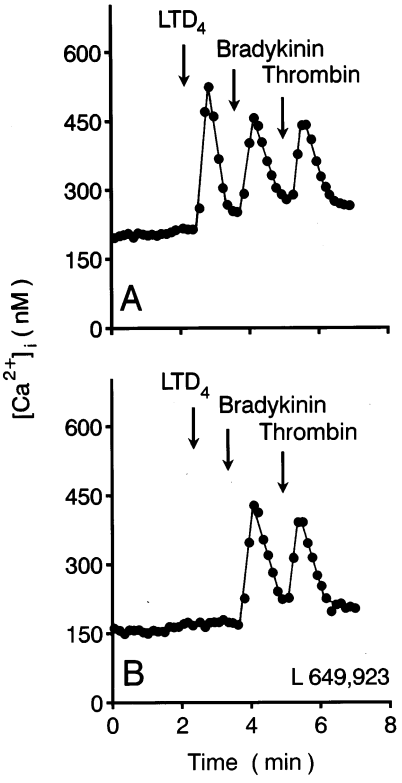


**Fig. 4.** Effect of the $LTD_4$-receptor antagonist, L649,923, on the $LTD_4$-induced increase in $[Ca^{2+}]_i$. The Ehrlich cells were suspended in standard medium containing 1 mM $Ca^{2+}$ and preincubated for 10–50 min. Measurements of $[Ca^{2+}]_i$ were obtained from *cell suspensions* (cytocrit 0.5%) using fura-2 and a fluorescence spectrophotometer. The $[Ca^{2+}]_i$ values were calculated from the 340 nm / 380 nm ratio values as described in Materials and Methods. (*A*) shows the increases in $[Ca^{2+}]_i$ after stimulation with $LTD_4$ (100 nM), bradykinin (10 μM) and thrombin (10 U/ml) added as indicated by the arrows. (*B*) shows that addition of L649,923 (1000 nM) abolishes the increase in $[Ca^{2+}]_i$ following stimulation with $LTD_4$ (100 nM), but has no effect on the bradykinin (10 μM) and thrombin (10 U/ml) induced responses. The agonists were added as indicated by the arrows. The data are representative of 2 experiments with addition of $LTD_4$ (100 nM) followed by bradykinin (10 μM) and thrombin (10 U/ml) and 1 experiment in which thrombin (10 U/ml) was added after $LTD_4$ (100 nM) before addition of bradykinin (10 μM).

this, at 20 μM L649,923 we find a slight increase in $[Ca^{2+}]_i$ before stimulation with agonists. It is noted that at 20 μM L649,923 the bradykinin response was also almost completely inhibited (*data not shown*). Thus, L649,923 seems to have some rather unspecific effects at higher concentrations.

### The $LTD_4$-induced Activation of $K^+$ and $Cl^-$ Channels is Independent of the Concomitant $[Ca^{2+}]$ Increase and Independent of Protein Kinase C

Figure 5 demonstrates the increase in $[Ca^{2+}]_i$ measured in a cell suspension ($Ca^{2+}$-free media, 2 mM EGTA with preincubation in $Ca^{2+}$-containing standard medium) after addition of either $LTD_4$ (100 nM, Fig. 5*A*) or ionomycin (2 μM, Fig. 5*B*). The $Ca^{2+}$ release is in both cases independent of the presence of the $Ca^{2+}$/calmodulin inhibitor pimozide. The $LTD_4$ (100 nM) and the ionomycin (2 μM)-induced increases in $[Ca^{2+}]_i$ are in the presence of pimozide (10 μM) 180 ± 71 nM ($n = 5$) and 1131 ± 485 nM ($n = 5$) and in the absence of pimozide 211 ± 90 nM ($n = 5$) and 374 ± 9 nM ($n = 3$), respectively. Figure 5 also demonstrates that whereas the ionomycin-induced cell shrinkage is inhibited in the presence of pimozide (the relative cell volume at the time of maximal shrinkage is 0.78 ± 0.02, $n = 3$ in control cells and 0.94 ± 0.02 $n = 3$ in the presence of pimozide) (Fig. 5*D*) the $LTD_4$-induced cell shrinkage is almost unaffected by the presence of pimozide (the relative cell volume at the time of maximal shrinkage is 0.82 ± 0.04, $n = 3$ in control cells and 0.89 ± 0.01, $n = 3$ in the presence of pimozide) (Fig. 5*C*). It should be mentioned that the biphasic volume response after addition of $LTD_4$ (Fig. 5*C*) reflects opening of $K^+$ and $Cl^-$ channels followed by activation of the $Na^+,K^+,2Cl^-$ cotransporter (Lambert, 1989) in analogy with the response to bradykinin, thrombin and histamine (*see* Hoffmann et al., 1993). Thus, the effect of the $Ca^{2+}$ ionophore-induced increase in $[Ca^{2+}]_i$ on the $K^+$ and $Cl^-$ channels involves a $Ca^{2+}$ calmodulin regulated system, whereas the activation of the $K^+$ and $Cl^-$ channels with $LTD_4$ predominantly does not. Since $LTD_4$ results in an increase in $[Ca^{2+}]_i$ it is likely that $Ca^{2+}$ activated $K^+$ channels contribute to the increase in $K^+$ permeability. The fact that we see almost no difference with pimozide probably reflects that the $K^+$ permeability after addition of $LTD_4$ is not rate limiting for the KCl loss, which is thus more likely limited by the $Cl^-$ permeability.

The rate of the RVD response is not affected by pretreatment for 15 min with chelerythrine (5 μM), which is sufficient to significantly inhibit protein kinase C dependent mechanisms in Ehrlich cells (Pedersen et al., 1996), neither in the presence nor in the absence of $LTD_4$ (100 nM). The rate of the RVD response was estimated at 110 ± 15 fl/min ($n = 3$) and 98 ± 2 fl/min ($n = 3$) in control cells with and without chelerythrine, respectively and at 256 ± 15 fl/min ($n = 3$) and 269 ± 91 fl/min ($n = 3$) in $LTD_4$-treated cells with and without chelerythrine, respectively. Thus neither the RVD response nor channel activation by $LTD_4$ seems to be dependent upon a protein kinase C.

### Effect of BAPTA-loading on RVD, $K^+$ and $Cl^-$ Fluxes

It has previously been demonstrated that the RVD response in $Ca^{2+}$-free $Cl^-$ medium is as effective as in a $Cl^-$ medium containing 1 mM $Ca^{2+}$ (Hoffmann et al., 1984). When the $K^+,Cl^-$ cotransporter is eliminated after substitution of $NO_3^-$ for all cellular and extracellular $Cl^-$ the

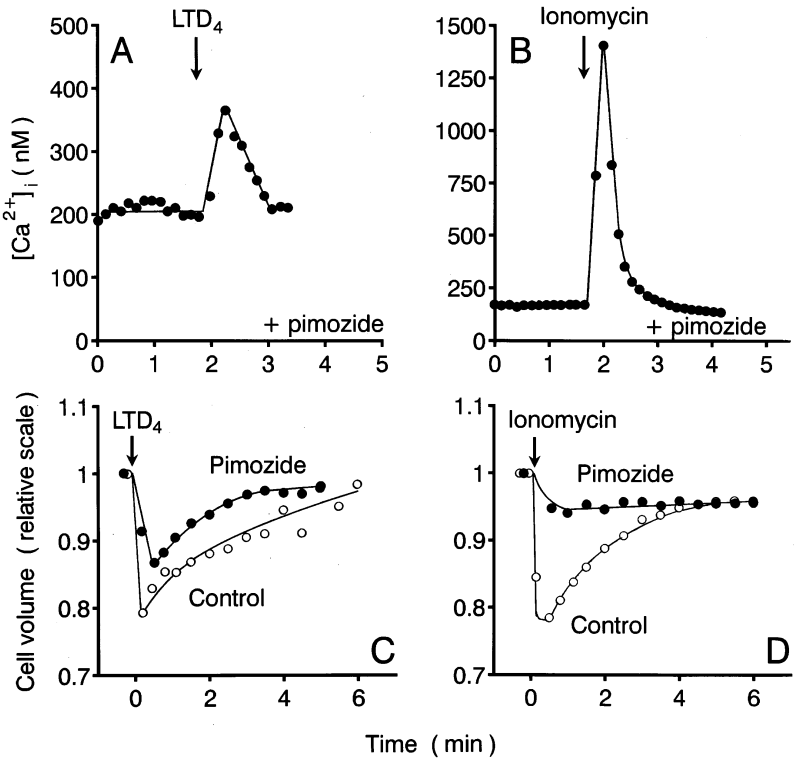


**Fig. 5.** Effect of the $Ca^{2+}$/calmodulin blocker pimozide on increases in $[Ca^{2+}]_i$ and cell shrinkage after addition of $LTD_4$ and ionomycin. (*A* and *B*): The cells were loaded with fura-2 as described in Materials and Methods and preincubated in $Ca^{2+}$-containing standard medium for 10–50 min. $[Ca^{2+}]_i$ was measured with time on the *cell suspension* in $Ca^{2+}$-free standard medium, 2 mM EGTA (cytocrit 0.5%) using a fluorescence spectrophotometer. Pimozide (10 μM) was added 2 min before stimulation. $LTD_4$ (100 nM, *A*) and ionomycin (2 μM, *B*) were added as indicated by the arrows. The resting level of $[Ca^{2+}]_i$ of approximately 200 nM is relatively high, but it should be noted that these cells have not been preincubated in the $Ca^{2+}$-free medium, and the resting level of $[Ca^{2+}]_i$ should thus be compared to the value estimated from cells suspended in $Ca^{2+}$-containing standard medium (160 ± 6 nM, $n = 73$). The calibration in the $Ca^{2+}$-free medium could furthermore be slightly affected by the relatively high level of background fluorescence at 380 nm in $Ca^{2+}$-free medium. The average resting level of $[Ca^{2+}]_i$ in $Ca^{2+}$-free medium (2 mM EGTA) was estimated to 23 ± 13 nM ($n = 186$) in single cell experiments, where the level of background fluorescence is lower. This can be compared to a resting level of 59 ± 2 nM ($n = 271$) estimated in single cells in standard medium, 1 mM $Ca^{2+}$ (*see* Table 2). (*C* and *D*): At time zero the cells (cytocrit 4%) were diluted 400-fold with isotonic $Ca^{2+}$-free standard medium, 2 mM EGTA containing pimozide (10 μM, closed symbols) and cell volume was followed with time using a Coulter counter. $LTD_4$ (100 nM, *C*) and ionomycin (2 μM, *D*) were added as indicated by the arrows. Control cells (open symbols) received no pimozide. The relative cell volume as estimated at the time of maximal cell shrinkage, following addition of $LTD_4$ and ionomycin, was in the *absence* of pimozide estimated at 0.82 ± 0.04 and 0.78 ± 0.02 ($n = 3$), respectively, and in the *presence* of pimozide at 0.89 ± 0.01 and 0.94 ± 0.02 ($n = 3$), respectively.

rate of the RVD response was found to be reduced by 25% in $Ca^{2+}$-free medium as compared to $Ca^{2+}$-containing medium (Kramhøft et al., 1986; Jørgensen et al., 1996). $Ca^{2+}$ depletion by pretreatment in $Ca^{2+}$-free media with EGTA and A23187, however, resulted in a 43% reduction in the rate of the RVD response (Hoffmann et al., 1984).

It has recently been demonstrated that buffering of $[Ca^{2+}]_i$ in the Ehrlich cells, by loading with the $Ca^{2+}$ chelator BAPTA, almost completely blocks the RVD response (Jørgensen et al., 1996). Loading of the Ehrlich cells with BAPTA also results in an intracellular acidification (S.F. Pedersen, *unpublished results: see* Table 2; *see also* Pedersen et al., 1994), which in itself inhibits the RVD response (Hoffmann et al., 1984). Preincubation of the Ehrlich cells in media at an extracellular pH ($pH_o$) of 8.3 after the cells have been loaded with BAPTA results in an intracellular pH ($pH_i$) value of 7.2, which is close to the $pH_i$ value in control cells without BAPTA in isotonic medium at $pH_o$ 7.4 ($pH_i$ 7.25) (S.F. Pedersen, *unpublished results; see* Table 2; *see also* Pedersen et al., 1994). Jørgensen et al. (1996), however, demonstrated that BAPTA significantly inhibited the RVD response also when $pH_i$ was kept at the normal value ($pH_i = 7.2$ at $pH_o$ 8.3). Thus, the inhibitory effect of BAPTA on the RVD response is not caused by a reduction in $pH_i$.

To test the effect of BAPTA more directly on the conductive $Cl^-$ efflux in hypotonic medium, $^{36}Cl^-$ efflux was measured (*i*) in media where gluconate was substituted for $Cl^-$ to avoid $Cl^-$ efflux via the anion exchanger

**Table 2.** Effect of buffering of cellular $Ca^{2+}$ with BAPTA on resting $[Ca^{2+}]_i$, $pH_i$, cell volume and ion content in cells suspended in isotonic standard medium (1 mM $Ca^{2+}$) and on the rate constant and efflux after cell swelling in hypotonic $Cl^-$-free gluconate medium (150 mOsm).

| | Control cells | BAPTA-treated cells | $pH_o$ |
|---|---|---|---|
| $[Ca^{2+}]_i$, nM | 59 ± 2 (271) | 26 ± 1 (66) | 7.4 |
| $pH_i$[a] | 7.25 | 7.00 | 7.4 |
| | 7.35 | 7.22 | 8.3 |
| Cell volume | | | |
| *fl* | 977 ± 19 (18) | 786 ± 22 (12) | 7.4 |
| *ml/g dry wt.* | 3.68 ± 0.10 (4) | 2.80 ± 0.07 (4) | 8.3 |
| Ion content, *μmol/g cell dry wt.* | | | |
| Chloride | 207 ± 2 (4) | 40 ± 3 (4) | 8.3 |
| Potassium | 769 ± 6 (4) | 595 ± 3 (4) | 8.3 |
| Sodium | 22 ± 7 (4) | 99 ± 8 (4) | 8.3 |
| Rate constant for efflux after hypotonic cell swelling, $min^{-1}$ | | | |
| Chloride (arachidonic acid sensitive[d]) | 0.34 ± 0.06 (3) | 0.14 ± 0.17[b] (3) | 8.3 |
| Potassium | 0.43 ± 0.08 (3) | 0.13 ± 0.05[c] (3) | 8.3 |
| Efflux after hypotonic cell swelling, *μmol/g dry wt*min*$^{-1}$ | | | |
| Chloride | 70 ± 13 (3) | 5 ± 7 (3) | 8.3 |
| Potassium | 330 ± 63 (3) | 74 ± 31 (3) | 8.3 |

Resting $[Ca^{2+}]_i$ was determined in *single cells* (*see* the legend to Fig. 1). $pH_i$ was determined using the pH-sensitive fluorescent probe BCECF (Pedersen et al., 1996). The cell volume at $pH_o$ 7.4 and 8.3 was estimated by the Coulter counter technique and as the water content, respectively. The cellular ion content was determined as described in Materials and Methods. The rate constants for $Cl^-$ and $K^+$ efflux after hypotonic cell swelling in $Cl^-$-free gluconate medium (150 mOsm) were determined from curves similar to Figs. 6 and 7, using Eqs. (1) and (2), respectively. The corresponding $Cl^-$ and $K^+$ efflux were calculated as the product of the rate constants and the corresponding ion content.

[a] S.F. Pedersen, *unpublished results, see* Pedersen et al., 1994.

[b] Not significantly different, $P > 0.5$.

[c] Significantly different, $P < 0.04$.

[d] Calculated as the difference between the rate constant measured in the presence (control cells: 0.18 ± 0.01; BAPTA-treated cells: 0.38 ± 0.1) and absence (control cells: 0.52 ± 0.07; BAPTA-treated cells: 0.52 ± 0.23) of arachidonic acid (200 μM).

(*ii*) in the presence of bumetanide (30 μM) to avoid $Cl^-$ efflux via the $K^+$,$Cl^-$ and the $Na^+$,$K^+$,$2Cl^-$ cotransport systems and (*iii*) in the presence of valinomycin (2.4 μM) to clamp the membrane potential on the $K^+$-equilibrium potential and to assure that the $Cl^-$ permeability was rate limiting. The swelling activated ''mini $Cl^-$ channel'' has previously been shown to be blocked by arachidonic acid (Lambert, 1987, 1991; Lambert & Hoffmann, 1994). Thus the arachidonic acid-sensitive $^{36}Cl^-$ efflux is taken as a measure of the $Cl^-$ efflux via the ''mini-$Cl^-$ channel.'' Figure 6 demonstrates that the swelling activated, arachidonic acid-sensitive $^{36}Cl^-$ efflux in BAPTA-loaded cells is slower than in control cells. Table 2 gives the $Cl^-$ content at the time of hypotonic exposure, the rate constant for the arachidonic acid-sensitive $Cl^-$ efflux as well as the initial, arachidonic acid-sensitive unidirectional $Cl^-$ efflux in hypotonic medium in control cells and in cells loaded with BAPTA. It is clear that BAPTA inhibits the swelling activated conductive $Cl^-$ flux. The $Cl^-$ conductance in hypotonic media at $pH_o$ 7.4 and $pH_i$ 7.3 has previously been estimated at 41 μS/cm$^2$ (Lambert et al., 1989). However, the absolute $Cl^-$ conductance and/or permeability was not estimated in the present experiments because the extracellular $Cl^-$ concentration in the gluconate medium was too low to measure and the $Cl^-$ gradient and $Cl^-$ equilibrium potential therefore impossible to determine.

The passive $K^+$ efflux from Ehrlich cells after cell swelling in hypotonic medium is also inhibited by preincubation with BAPTA, as seen in Fig. 7 and Table 2. Figure 7 demonstrates the $^{86}Rb^+$ efflux after hypotonic cell swelling, whereas Table 2 gives the $K^+$ content at the time of hypotonic exposure, the rate constant for $K^+$ efflux as well as the initial unidirectional $K^+$ efflux in control cells and in BAPTA-treated cells in the presence of bumetanide (30 μM). It is assumed that $^{86}Rb^+$ can be regarded as a tracer for $K^+$. Unless the membrane potential is hyperpolarized in BAPTA-loaded cells, which is highly unlikely, then the conductances in the BAPTA-loaded cells in hypotonic medium is lower than the conductance in hypotonic medium without BAPTA at $pH_o$ 7.4 as well as at $pH_o$ 8.3.

Addition of gramicidin in $Na^+$-free hypotonic media has previously been demonstrated to accelerate the RVD response in Ehrlich cells (Hoffmann et al., 1986) in accordance with the notion that the $K^+$ conductance is lower than the $Cl^-$ conductance in osmotically swollen cells (Lambert et al., 1989). Jørgensen et al. (1996) de-

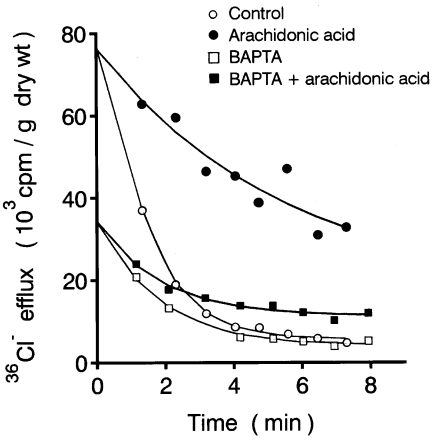


**Fig. 6.** Effect of buffering cellular $Ca^{2+}$ with BAPTA on the swelling-activated $^{36}Cl^-$ efflux. Ehrlich cells were equilibrated with $^{36}Cl^-$ in standard NaCl medium, $pH_o$ 8.3. The cytocrit was 0.8% for control cells and 0.4% for cells to be loaded with BAPTA. At time zero the cells were transferred to hypotonic (150 mOsm), $Cl^-$-free gluconate medium containing bumetanide (30 μM) and valinomycin (2.4 μM). The $^{36}Cl^-$ efflux was followed with time. The final experimental cytocrit was 4%. Bumetanide was added to prevent $^{36}Cl^-$ efflux via the $K^+$,$Cl^-$-cotransporter and the $Na^+$,$K^+$,$2Cl^-$-cotransporter, whereas valinomycin was added in order to clamp the membrane potential at the $K^+$ equilibrium potential. $^{36}Cl^-$ efflux from control cells (circles) and from BAPTA-loaded cells (squares) are shown as the loss in cellular $^{36}Cl^-$ activity (cpm/g cell dry wt) in the presence (closed symbols) and in the absence (open symbols) of 200 μM arachidonic acid. Arachidonic acid was added to inhibit the swelling-activated ''mini-$Cl^-$-channel.'' The data are from a single representative experiment of four experiments.

monstrated that gramicidin is able to accelerate the RVD response in low $Na^+$ medium after loading of the cells with BAPTA, indicating that the $K^+$ permeability is still rate limiting for the RVD response after BAPTA loading.

It should be noted that loading Ehrlich cells for 35 min with 50 μM BAPTA-AM reduces the cell volume at $pH_o$ 7.4 from 977 ± 19 fl ($n$ = 18) to 786 ± 22 fl ($n$ = 12) (estimated by the Coulter counter technique) and at $pH_o$ 8.3 from 3.68 ± 0.1 ml/g dry wt ($n$ = 4) to 2.8 ± 0.07 ml/g dry wt ($n$ = 4) (estimated as the reduction in the water content) (Table 2). Furthermore, at $pH_o$ 8.3 it was estimated that BAPTA-AM reduced the $Cl^-$ and the $K^+$ content from 207 ± 2 μmol/g dry wt ($n$ = 4) and 769 ± 6 μmol/g dry wt ($n$ = 4) to 40 ± 3 μmol/g dry wt ($n$ = 4) and 595 ± 4 μmol/g dry wt ($n$ = 4), respectively, and increased the $Na^+$ content from 22 ± 7 μmol/g dry wt to 99 ± 8 μmol/g dry wt (*see* Table 2). Thus, $Ca^{2+}$ buffering with BAPTA results in net loss of KCl and cell water. This is likely to result from the fact that the low resting level of $[Ca^{2+}]_i$ in BAPTA-loaded cells (*see* Table 2) stimulates the $K^+$,$Cl^-$ cotransporter, i.e., KCl net loss (Kramhøft et al., 1986) and inhibits the $Na^+$,$K^+$,$2Cl^-$ cotransporter, i.e., KCl reuptake (L. Jakobsen, *unpublished results*).

In summary, buffering $[Ca^{2+}]_i$ in Ehrlich cells with BAPTA reduces the KCl content, the cell volume, and it reduces the swelling-activated, arachidonic acid-sensitive $Cl^-$ efflux with 93% and the swelling activated $K^+$ efflux by 78% ($pH_o$ 8.3, Table 2). Since $pH_i$ in the BAPTA-loaded cells is 7.2 compared to 7.35 in control cells ($pH_o$ 8.3) a slight pH effect on the swelling-activated transport systems cannot be excluded.

## The Effect of $Ca^{2+}$ Buffering or Inhibition of $Ca^{2+}$/Calmodulin on the $LTD_4$ Induced Cell Shrinkage

$LTD_4$ accelerates the rate of the RVD response at concentrations ⩾ 3 nM (*see* Table 1), and at concentrations > 60 nM the acceleration is highly significant (Lambert et al., 1987). Figure 8 demonstrates that if $[Ca^{2+}]_i$ is buffered with BAPTA there is no acceleration of the RVD response after addition of a high concentration of $LTD_4$ (100 nM). It should be noted that addition of $LTD_4$ to control cells 6 min after hypotonic exposure still accelerates the RVD response significantly (Lambert, 1989) and that addition of gramicidin to BAPTA-loaded cells can accelerate the RVD response (Jørgensen et al., 1996), i.e., the lack of effect of $LTD_4$ in the BAPTA-loaded cells is neither due to a reduced potency of $LTD_4$ when added later than 1 min after the hypotonic exposure nor due to lack of an outward KCl gradient. Furthermore, addition of 100 nM $LTD_4$ does not cause an isotonic volume reduction of BAPTA-loaded cells (2 experiments, *data not shown*). Since stimulation with 100 nM $LTD_4$ in $Ca^{2+}$-containing medium results in $Ca^{2+}$ release as well as $Ca^{2+}$ influx an increased acidification in

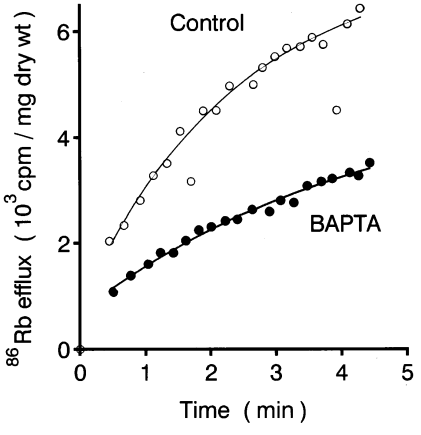


**Fig. 7.** Effect of buffering cellular $Ca^{2+}$ with BAPTA on the swelling-activated $^{86}Rb^+$ efflux. Ehrlich cells were equilibrated with $^{86}Rb^+$ in standard NaCl medium, $pH_o$ 8.3. The cytocrit was 0.8% for control cells and 0.4% for cells to be loaded with BAPTA. At time zero the cells were transferred to hypotonic (150 mOsm), $Cl^-$-free gluconate medium containing bumetanide (30 μM). The $^{36}Cl^-$ efflux was followed with time. The final experimental cytocrit was 4%. Bumetanide was added to prevent $^{86}Rb^+$ efflux via the $K^+$,$Cl^-$ cotransporter and the $Na^+$,$K^+$,$2Cl^-$ cotransporter. $^{86}Rb^+$ efflux from control cells (open circles) and from BAPTA-loaded cells (closed circles) are shown as the gain in extracellular $^{86}Rb^+$ activity (cpm/mg dry wt). The data are from a single representative experiment of three experiments.

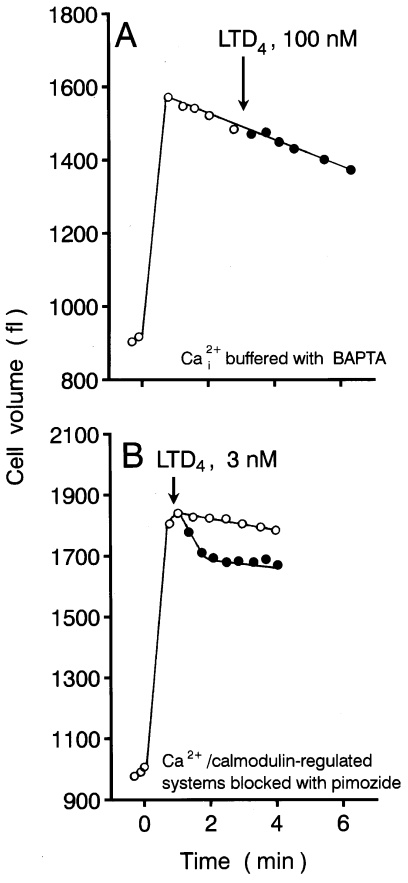


**Fig. 8.** Effect of buffering cellular $Ca^{2+}$ with BAPTA or inhibition of $Ca^{2+}$/calmodulin on the $LTD_4$-induced acceleration of the RVD response. The cells were treated as described in the legend to Fig. 2*B*, except that the extracellular $Ca^{2+}$ concentration in *A* was 1 mM. BAPTA-loaded cells were treated as described in Materials and Methods. *A* shows the effect of $LTD_4$ (100 nM, closed symbols) added to BAPTA-loaded cells as indicated by the arrow. *B* shows the effect of $LTD_4$ (3 nM, closed symbols) added as indicated by the arrow in the presence of pimozide (10 μM) ($Ca^{2+}$-free medium, 2 mM EGTA). Pimozide was added at the time of hypotonic exposure to block $Ca^{2+}$/calmodulin-regulated systems. Control cells (open symbols) received no $LTD_4$. The data are from a single experiment, representative of a total of three identical sets of experiments.

the presence of BAPTA might be expected due to release of protons in the chelation process similar to what is seen with EGTA (Marks & Maxfield, 1991). We therefore tried stimulation of BAPTA-loaded cells with 3 nM $LTD_4$ in $Ca^{2+}$-free medium (2 mM EGTA), where the increase in $[Ca^{2+}]_i$ is smaller. This likewise did not cause any cell shrinkage (2 experiments, *data not shown*). Figure 8*B* demonstrates that inhibition of $Ca^{2+}$/calmodulin-regulated systems by addition to pimozide (10 μM) on the other hand does not prevent acceleration of the rate of the RVD response after addition of a low concentration of $LTD_4$ (3 nM). Similar results have previously been demonstrated for higher concentrations of $LTD_4$ (Lambert, 1989; Lauritzen et al., 1993). The fact that RVD is completely blocked by 10 μM pimozide (*see* Fig. 8*B* and Hoffmann et al., 1984) could thus reflect an inhibition by pimozide of the $LTD_4$ synthesis rather than of the $LTD_4$-induced channel opening. The specificity of pimozide towards $Ca^{2+}$/calmodulin is probably not high enough to allow further conclusions on this point.

## Desensitization of the $LTD_4$ Receptor

Desensitization of leukotriene receptors is observed in several cells and tissues (Chan et al., 1994; Winkler et al., 1988; *see* Lambert, 1994). Figure 9*A* demonstrates that addition of $LTD_4$ (100 nM) results in an increase in $[Ca^{2+}]_i$ (measured on a cell suspension), whereas the subsequent additions (1.4 and 2.9 min after the first $LTD_4$ addition) have no effect. Stimulation with another agonist like bradykinin, however, still results in a significant

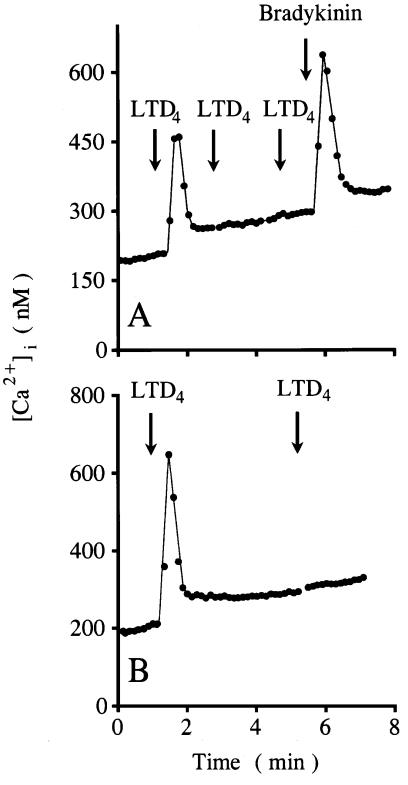


**Fig. 9.** Desensitization of the $LTD_4$ receptor, the effect of successive additions of $LTD_4$ on $[Ca^{2+}]_i$. The cells were treated as described in the legend to Table 1 and $[Ca^{2+}]_i$ was followed in the *cell suspension* in standard medium (1 mM $Ca^{2+}$) using fura-2 and a fluorescence spectrophotometer (*see* Materials and Methods). *A* $LTD_4$ (100 nM) and bradykinin (10 μM) were added as indicated by the arrows. *B* $LTD_4$ (100 nM) was added as indicated by the arrows. The data are from two individual experiments, representative of a total of five experiments, where the $LTD_4$ was added with varying time intervals between the additions.

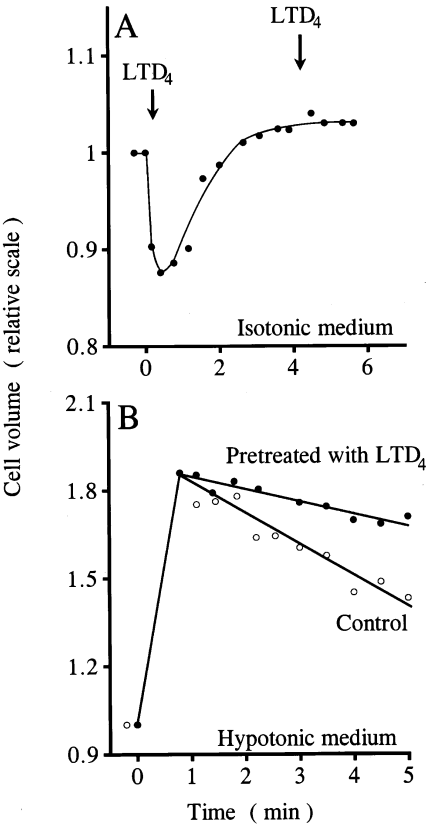


**Fig. 10.** Effect of desensitization of the $LTD_4$ receptor on the $LTD_4$-induced KCl loss and on the RVD response. *A* The cells were preincubated in isotonic standard medium for 10–50 min. At time zero the cells (cytocrit 4%) were diluted 400-fold into isotonic standard medium and cell volume was followed with time using a Coulter counter. $LTD_4$ (100 nM) was added as indicated by the arrows. The data are from a single experiment, representative of a total of three sets of experiments. *B* The cell suspension, preincubated in isotonic standard medium for 10–50 min (250,000 cells/ml), was at time zero diluted with buffered water (50 ml buffered water was added to 50 ml suspension, final osmolarity 150 mOsm). Cell volume was followed with time using a Coulter counter. To desensitize the $LTD_4$ receptor, 100 nM $LTD_4$ was added to the cells 7 min before the hypotonic shock (closed symbols). The rate of the regulatory volume decrease, calculated as the cell volume recovery between 1 min and 5 min after hypotonic exposure, was estimated at 97 ± 7 fl/min in control cells and at 43 ± 6 fl/min ($n = 7$) in cells pretreated for 5 to 8 min with 100 nM $LTD_4$. In the presence of the $Na^+$, $K^+$, $Cl^-$ cotransport inhibitor bumetanide (10 μM) the rate of water loss was estimated at 41 ± 7 fl/min in cells pretreated with $LTD_4$ and 75 ± 1 in control cells ($n = 4$).

increase in $[Ca^{2+}]_i$. This indicates that the receptor for $LTD_4$ is desensitized. Experiments performed with 4.2 min intervals between additions of $LTD_4$ gave similar results (Fig. 9*B*).

### Can the $K^+$ and $Cl^-$ Channels be Activated by Cell Swelling in the Period Where the $LTD_4$ Receptor is Desensitized?

Figure 10*A* demonstrates that the $LTD_4$-induced cell shrinkage is absent after a second addition of $LTD_4$ 4 min after the first addition, indicating that also the $LTD_4$-induced KCl loss shows a desensitization. Figure 10*B* compares the RVD response in 150 mOsm medium with and without 7 min preincubation with $LTD_4$ (100 nM). It is seen that pretreatment with $LTD_4$ clearly inhibits the RVD response. In seven paired experiments it was estimated that the rate of the RVD response in the hypotonic medium (150 mOsm) was reduced to 46 ± 7% ($n = 7$) of the control value when the cells were preincubated with 100 nM $LTD_4$ for 5 to 8 min before the hypotonic exposure (*see* legend to Fig. 10). It has previously been demonstrated that the $Na^+,K^+,2Cl^-$ cotransport system in Ehrlich cells is activated after stimulation with the $Ca^{2+}$ ionophore A23187 plus $Ca^{2+}$ (*see* Hoffmann et al., 1986) or by $Ca^{2+}$ mobilizing agonists (*see* Hoffmann et al., 1993), and that the activity of the $Na^+,K^+,2Cl^-$ cotransport system declines during the 6–8 min following stimulation, whereupon the activity reaches resting levels (Jakobsen, Jensen & Hoffmann, 1994). Thus, some residual cotransport activity during the RVD measurements could give a false impression of inhibition. However, if bumetanide (10 μM) was included in the hypotonic solution in order to avoid any influx via the $Na^+,K^+,2Cl^-$ cotransport system, preincubation with 100 nM $LTD_4$ for 5 to 7 min before the hypotonic exposure reduced the subsequent RVD response to 51 ± 10% of the control value ($n = 4$). The experiments, therefore, show that the swelling-induced activation of osmolyte transporting systems seems to be inhibited when the $LTD_4$ receptor is desensitized.

## Discussion

### Independence of the $LTD_4$-induced Increase in $[Ca^{2+}]_i$ and the $LTD_4$-induced $K^+$ and $Cl^-$ Channel Activation

Stimulation of Ehrlich cells with $LTD_4$ results in a transient increase in $[Ca^{2+}]_i$ already after addition of $LTD_4$ in the nM range. The rise in $[Ca^{2+}]_i$ has been measured in cell suspensions as well as in single cells (Fig. 1, Table 1; Lambert, 1994). This is in agreement with findings in several other cell types (*see* Introduction). In the absence of extracellular $Ca^{2+}$ the response is strongly reduced (*see* Table 1) indicating that part of the $LTD_4$-induced increase in $[Ca^{2+}]_i$ is caused by $Ca^{2+}$ influx. The concentration of $LTD_4$ required for half maximal increase in $[Ca^{2+}]_i$ $EC_{50}$ in $Ca^{2+}$-containing media and in $Ca^{2+}$-free media has recently been estimated at 10 ± 1 nM and 19 ± 3 nM, respectively (Pedersen et al., 1995). The $EC_{50}$ for $LTD_4$-induced $Ca^{2+}$ influx was estimated at 6 ± 2 nM (Pedersen et al., 1995). Removal of divalent cations has been demonstrated to lower the affinity of the $LTD_4$ receptor for $LTD_4$ in human THP-1 cells (Rochette et al., 1993), which could explain the increase in $EC_{50}$ in

$Ca^{2+}$-free medium. Alternatively, the higher $EC_{50}$ in $Ca^{2+}$-free medium could also reflect that a physiological transmembrane $Ca^{2+}$ gradient is important for G-protein function as demonstrated by Fan and coworkers (1995) in their study of reconstituted $G_s$ and adenylate cyclase from bovine brain.

At 3 nM $LTD_4$, a significant response is seen at 1 mM external $Ca^{2+}$ whereas no detectable change in $[Ca^{2+}]_i$ is seen in $Ca^{2+}$-free media with 2 mM EGTA (Table 1; Fig. 2), in agreement with the results reported for THP-1 cells (Chan et al., 1994). Baud et al. (1987) have reported significant increases in $[Ca^{2+}]_i$ after stimulation of HL-60 cells with even lower concentrations of $LTD_4$ (0.15 nM), in their study the increase in $[Ca^{2+}]_i$ was dependent upon influx of $Ca^{2+}$ from the medium. We have in agreement with these results found a significant increase in $[Ca^{2+}]_i$ after stimulation of Ehrlich cells with 1 nM $LTD_4$ in $Ca^{2+}$-containing medium (Pedersen et al., 1995), but we have not further investigated the effect of lower $LTD_4$ concentrations (*below* 1 nM). Despite the lack of a measurable increase in $[Ca^{2+}]_i$, 3 nM $LTD_4$ can, however, still accelerate the RVD response in Ehrlich cells in $Ca^{2+}$-free media with 2 mM EGTA (*see* Fig. 2*B* and Table 1). This indicates that the channel activation by $LTD_4$ is independent of any detectable increase in $[Ca^{2+}]_i$, favoring the idea of a more direct activation of the channels by $LTD_4$. This is further supported by the finding that inhibition of protein kinase C has no effect on either the rate of the RVD response or the $LTD_4$-induced acceleration of the RVD response (*see* Results). An additional argument for the notion that the $K^+$ and $Cl^-$ channels activated by $LTD_4$ are different from the channels activated by $Ca^{2+}$ is that the $LTD_4$-induced activation of the channels is not affected by the calmodulin inhibitor pimozide (Fig. 5*C*), whereas the $Ca^{2+}$-induced activation of $K^+$ and $Cl^-$ channels is strongly inhibited (Fig. 5*D*), consistent with previous findings (Hoffmann et al., 1986). Moreover, it has recently been demonstrated that the channels activated during RVD are insensitive to charybdotoxin whereas the $Ca^{2+}$-activated $K^+$ channels are blocked by charybdotoxin (Harbak & Simonsen, 1995; Jørgensen et al., 1996). Similarly, the accelerating effect of $LTD_4$ on the RVD response is also unaffected by pimozide (Fig. 8; Lambert, 1989; Lauritzen et al., 1993).

### Could the Activation of the $K^+$ and $Cl^-$ Channels During RVD and After Addition of Low Concentrations of $LTD_4$ Result from an Undetectable Localized Increase in $[Ca^{2+}]_i$?

It has previously been demonstrated that Ehrlich cells can volume regulate in hypotonic media without any detectable increase in $[Ca^{2+}]_i$ (*see* Jørgensen et al., 1996). Buffering $[Ca^{2+}]_i$ by BAPTA, however, inhibits the RVD response at $pH_o$ 7.4, $pH_i$ 6.99 as well as at $pH_o$ 8.3, $pH_i$ 7.3 (Jørgensen et al., 1996). The swelling-induced activation of the $Cl^-$ and $K^+$ channels is inhibited in BAPTA-loaded cells as seen from Table 2 and from the $^{36}Cl^-$-efflux measurements (Fig. 6) and the $^{86}Rb^+$ efflux measurements (Fig. 7). The arachidonic acid-sensitive $Cl^-$ efflux during RVD is reduced from 70 μmol/g dry wt · min in control cells to 5 μmol/g dry wt · min after BAPTA loading (*see* Table 2) and the $K^+$ efflux (measured as $^{86}Rb^+$ efflux) is reduced from 330 μmol/g dry wt · min during control RVD to 74 μmol/g dry wt · min after BAPTA loading (*see* Table 2). These findings are taken to indicate that a certain level of $[Ca^{2+}]_i$ is necessary for activation of the channels, or alternatively that a small localized (and undetectable) increase in $[Ca^{2+}]_i$ is normally taking place during RVD. Many of the enzymes involved in the synthesis of $LTD_4$ are $Ca^{2+}$ dependent (*see* e.g., Lambert, 1994). Thus, the inhibition seen after BAPTA loading could be in the synthesis sequence for $LTD_4$ as well as in the activation of the channels. The inhibition of the RVD response in BAPTA-loaded cells cannot be lifted by addition of $LTD_4$ (Fig. 8*A*) and similarly no cell shrinkage is recorded either after addition of 100 nM $LTD_4$ to BAPTA-loaded cells suspended in isotonic $Ca^{2+}$-containing medium or after addition of 3 nM $LTD_4$ to BAPTA-loaded cells suspended in $Ca^{2+}$-free medium (2 mM EGTA). This suggests that it is the actual activation of the channels by $LTD_4$ which is impaired in the BAPTA-loaded cells. Thus it cannot be excluded that the opening of $K^+$ and $Cl^-$ channels during the RVD response as well as after addition of low concentrations of $LTD_4$ could result from a small localized undetectable increase in $Ca^{2+}$. It is noted, however, that some rather unspecific effects of BAPTA, not directly related to the chelation of $Ca^{2+}$, have been observed. The BAPTA-loading or the formaldehyde released from hydrolysis of the AM-ester groups have been reported to cause a reduction of intracellular ATP levels in rat parotid cells and human red cells, respectively (Tojyo & Matsumoto, 1990, Garcia-Sancho, 1985), and BAPTA-loading has been found to translocate and inhibit protein kinase C in macrophages (Dieter, Fitzke & Duyster, 1993), to affect arachidonate metabolism in endothelial cells (Boeynaems et al., 1993) and to antagonize binding of $IP_3$ to its receptor (Richardson & Taylor, 1993).

### The Leukotriene $D_4$ Receptor

The leukotriene receptor antagonist L649,923 inhibits the $LTD_4$-induced ion channel activation as well as the $LTD_4$-induced $Ca^{2+}$ transients. Figure 3 demonstrates the dose-dependent inhibition by L649,923 of the $LTD_4$-induced acceleration of the RVD response with a half maximal effect around 250 nM L649,923, i.e., in the concentration range reported to inhibit the leukotriene receptor (Jones et al., 1986). Figure 4 confirms that L649,923

is specific for the $LTD_4$ receptor and does not affect phospholipase C or other steps in the inositol phosphate signalling pathway, because the changes in $[Ca^{2+}]_i$ after addition of the agonists bradykinin and thrombin are unaffected by the drug. These results indicate that although the mechanism of channel activation by $LTD_4$ seems to be different from the mechanism leading to changes in $[Ca^{2+}]_i$ both effects are likely to be mediated via a $LTD_4$ receptor. The $LTD_4$ receptor seems to be specific for $LTD_4$, because $LTB_4$, $LTC_4$ and $LTE_4$ all are unable to mimic the effect of $LTD_4$ on volume changes (Lambert, 1989) and on the increase in $[Ca^{2+}]_i$ (Pedersen et al., 1995). Whether the Ehrlich cells might have two separate $LTD_4$ receptor types or alternatively a common $LTD_4$ receptor coupled via different types of G proteins to the $K^+$ and $Cl^-$ channel activating pathway and the $Ca^{2+}$-mobilizing pathway is currently under investigation. That $LTD_4$ can activate at least two types of G proteins has been described by Sjölander and coworkers (1990) for the $LTD_4$ receptor in an intestinal epithelial cell line. One of the G proteins was pertussis-toxin sensitive, the other was not. The $Ca^{2+}$-influx was regulated by the pertussis-toxin sensitive G protein, release of $Ca^{2+}$ by the pertussis-toxin insensitive (Sjölander et al., 1990). The partial inhibition of the $LTD_4$ accelerated RVD response in Ehrlich cells by pertussis toxin described by Lambert (1989) does not permit any conclusions with respect to the possible involvement of different G proteins.

### Desensitization of the $LTD_4$ Receptor Results in a Strong Inhibition of the RVD Response

We found a desensitization of the $LTD_4$ receptor after 2–4 min stimulation with 100 nM $LTD_4$ measured as a desensitization of the $Ca^{2+}$ response (Fig. 9*A* and *B*) as well as of the $LTD_4$-induced cell shrinkage (Fig. 10*A*). The effect is specific for the $LTD_4$ receptor as stimulation with the agonist bradykinin still results in a normal increase in $[Ca^{2+}]_i$ in the period where the cells do not respond to $LTD_4$ (*see* Fig. 9*A*). Provided the normal RVD response as suggested is caused by $LTD_4$, which acting as a ''local hormone'' is opening $K^+$ and $Cl^-$ channels, it is predicted that a normal RVD response will also be desensitized after pretreatment of the cells with $LTD_4$. This is actually found to be the case as seen from Fig. 10. The rate of the RVD response was reduced to 46 ± 6% ($n = 7$) of the control values after $LTD_4$ pretreatment. The results are similar in the absence and in the presence of bumetanide, added in order to ensure that the $Na^+$,$K^+$,2$Cl^-$ cotransporter was not causing reuptake of KCl and thereby mimicking an inhibition of the RVD response. This desensitization of the RVD response after pretreatment with $LTD_4$ is, therefore, taken as a strong argument for the hypothesis that a $LTD_4$ receptor is involved in the swelling-induced activation of $K^+$ and $Cl^-$ channels. It is, as a working hypothesis, tempting to suggest that we are dealing either with new types of receptor-gated ion channels, or with channels controlled via G proteins activated by the $LTD_4$ receptor.

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