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Leukotriene D_4-induced Ca^{2+} Mobilization in Ehrlich Ascites Tumor Cells

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Abstract. Stimulation of Ehrlich ascites tumor cells with leukotriene D_4 (LTD_4) within the concentration range 1–100 nM leads to a concentration-dependent, transient increase in the intracellular, free Ca^{2+} concentration, $[Ca^{2+}]_i$. The Ca^{2+} peak time, i.e., the time between addition of LTD_4 and the highest measured $[Ca^{2+}]_i$ value, is in the range 0.20 to 0.21 min in ten out of fourteen independent experiments. After addition of a saturating concentration of LTD_4 (100 nM), the highest measured increase in $[Ca^{2+}]_i$ in Ehrlich cells suspended in Ca^{2+}-containing medium is 260 ± 14 nM and the EC_{50} value for LTD_4-induced Ca^{2+} mobilization is estimated at 10 nM. Neither the peptido-leukotrienes LTC_4 and LTE_4 nor LTB_4 are able to mimic or block the LTD_4-induced Ca^{2+} mobilization, hence the receptor is specific for LTD_4. Removal of Ca^{2+} from the experimental buffer significantly reduces the size of the LTD_4-induced increase in $[Ca^{2+}]_i$. Furthermore, depletion of the intracellular Ins(1,4,5)P_3-sensitive Ca^{2+} stores by addition of the ER-Ca^{2+}-ATPase inhibitor thapsigargin also reduces the size of the LTD_4-induced increase in $[Ca^{2+}]_i$ in Ehrlich cells suspended in Ca^{2+}-containing medium, and completely abolishes the LTD_4-induced increase in $[Ca^{2+}]_i$ in Ehrlich cells suspended in Ca^{2+}-free medium containing EGTA. Thus, the LTD_4-induced increase in $[Ca^{2+}]_i$ in Ehrlich cells involves an influx of Ca^{2+} from the extracellular compartment as well as a release of Ca^{2+} from intracellular Ins(1,4,5)P_3-sensitive stores. The Ca^{2+} peak times for the LTD_4-induced Ca^{2+} influx and for the LTD_4-induced Ca^{2+} release are recorded in the time range 0.20 to 0.21 min in four out of five experiments and in the time range 0.34 to 0.35 min in six out of eight experiments, respectively. Stimulation with LTD_4 also induces a transient increase in Ins(1,4,5)P_3 generation in the Ehrlich cells, and the Ins(1,4,5)P_3 peak time is recorded in the time range 0.27 to 0.30 min. Thus, the Ins(1,4,5)P_3 content seems to increase before the LTD_4-induced Ca^{2+} release from the intracellular stores but after the LTD_4-induced Ca^{2+} influx. Inhibition of phospholipase C by preincubation with U73122 abolishes the LTD_4-induced increase in Ins(1,4,5)P_3 as well as the LTD_4-induced increase in $[Ca^{2+}]_i$, indicating that a U73122-sensitive phospholipase C is involved in the LTD_4-induced Ca^{2+} mobilization in Ehrlich cells. The LTD_4-induced Ca^{2+} influx is insensitive to verapamil, gadolinium and SK&F 96365, suggesting that the LTD_4-activated Ca^{2+} channel in Ehrlich cells is neither voltage gated nor stretch activated and most probably not receptor operated. In conclusion, LTD_4 acts in the Ehrlich cells via a specific receptor for LTD_4, which upon stimulation initiates an influx of Ca^{2+}, through yet unidentified Ca^{2+} channels, and an activation of a U73122-sensitive phospholipase C, Ins(1,4,5)P_3 formation and finally release of Ca^{2+} from the intracellular Ins(1,4,5)P_3-sensitive stores.

Key words: Leukotriene D_4 receptor — Phospholipase C — Ins(1,4,5)P_3 — Ca^{2+} mobilization — Thapsigargin — U73122

Introduction

The leukotrienes LTB_4, LTC_4, LTD_4 and LTE_4 are synthesized in mammalian cells from the polyunsaturated fatty acid arachidonic acid (*see* Samuelsson et al., 1987; Holtzman, 1992) and they are biologically very potent substances. LTB_4 stimulates chemokinesis and chemotaxis of neutrophils, eosinophils, as well as neutrophil adherence, aggregation and lysosomal degradation, whereas the peptidoleukotrienes LTC_4, LTD_4 and LTE_4 are known to produce broncho- and vasoconstriction, induce edema, increase the vascular permeability and enhance mucus secretion in the lungs (*see* Nicosia & Pa-

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trono, 1989; Brain & Williams, 1990; Serhan, 1991; Hay, Torphy & Undem, 1995).

LTD_4 is believed to be the most potent of the peptidoleukotrienes, and it is generally agreed that the LTD_4-induced intracellular signaling involves an increase in the intracellular, free Ca^{2+} concentration ($[Ca^{2+}]_i$) (*see* Crooke et al., 1989; Sjölander & Grönroos, 1994). In Ehrlich ascites tumor cells LTD_4 is shown to induce cell shrinkage and KCl loss apparently from an increase in K^+ and Cl^- permeabilities (Lambert et al., 1987; Lambert 1987; Lambert, 1989) and to induce a transient increase in $[Ca^{2+}]_i$ (Jørgensen, Lambert & Hoffmann, 1996; *see* Lambert, 1994, and Hoffmann & Dunham, 1995). In addition, LTD_4 is found to increase the taurine leak permeability in Ehrlich cells (Lambert & Hoffmann, 1993). Since LTD_4 also is an important second messenger during cell volume regulation in Ehrlich cells (*see* Lambert, 1994; Hoffmann & Dunham, 1995), the present investigation was initiated in order to gain further information of LTD_4 signaling properties and the LTD_4 receptor in these cells.

The LTD_4-induced increase in $[Ca^{2+}]_i$ seems in several cell types to involve an influx of Ca^{2+} from the extracellular compartment as well as a release of Ca^{2+} from internal stores (Saussy et al., 1989; Chan et al., 1994; Jørgensen et al., 1996). The Ca^{2+} mobilizing effect of LTD_4 is thought to be mediated via interaction of LTD_4 with a specific receptor (Sarau et al., 1987), which seems to be coupled to at least two types of G-proteins (Crooke et al., 1989; Sjölander et al., 1990). One of these G-proteins is presumed to activate phospholipase C (PLC) resulting in an increased $Ins(1,4,5)P_3$ formation and subsequently Ca^{2+} release, whereas the other G-protein is sensitive to pertussis toxin and believed to be involved in activation of the Ca^{2+} influx pathway (Saussy et al., 1989; Sjölander et al., 1990).

In the present study, we characterize the nature of the LTD_4-induced Ca^{2+} mobilization in Ehrlich ascites tumor cells. We have followed the LTD_4-induced increase in $[Ca^{2+}]_i$ in the presence and absence of external Ca^{2+}, after depletion of the intracellular $Ins(1,4,5)P_3$-sensitive Ca^{2+} stores with thapsigargin, and after inhibition of (i) nonselective stretch-activated cation (SA-cat) channels, which has previously been demonstrated to be present in Ehrlich cells (Christensen & Hoffmann, 1992), (ii) voltage gated Ca^{2+} (VOC) channels or (iii) receptor activated Ca^{2+} (ROC) channels. The data indicate, that LTD_4-induced Ca^{2+} mobilization in the Ehrlich cells demands PLC activity and involves both an influx of Ca^{2+} as well as an $Ins(1,4,5)P_3$-mediated release of Ca^{2+} from intracellular stores. The peak in $[Ca^{2+}]_i$ due to the increased Ca^{2+} influx appears to occur before the peak in $[Ca^{2+}]_i$ due to release of Ca^{2+} from the intracellular stores and before the maximal increase in the $Ins(1,4,5)P_3$ content.

A part of this investigation has been presented in an abstract form at the Scandinavian Physiological Society meeting in Copenhagen 1995 (Pedersen et al., 1995).

Materials and Methods

Cell Suspension

Ehrlich ascites tumor cells (hyperdiploid strain) were maintained in NMRI mice (25–30 g) by weekly intraperitoneal transplantation. One week after transplantation the mice were sacrificed and the cells harvested in standard incubation medium as described by Hoffmann et al. (1986). Subsequently, the cells were resuspended at a cytokrit of 4% in standard medium and incubated 30 min before commencing the experiments. During this period, loading of the cells with fura-2 was initiated (*see below*). When using Ca^{2+}-free medium, the loaded cells were washed additionally prior to the experiment and incubated for another 10 to 50 min. The experiments were all executed at 37°C.

Incubation Media

Standard incubation medium (300 mOsm) was composed as follows (in mM): 150 Na^+, 5 K^+, 1 Mg^{2+}, 1 Ca^{2+}, 150 Cl^-, 1 SO_4^{2-}, 1 PO_4^{3-}, 3.3 MOPS, 3.3 TES, 5 HEPES, pH 7.4. In low Ca^{2+} medium, the $[Ca^{2+}]$ was reduced to 0.1 mM. In Ca^{2+}-free medium, addition of Ca^{2+} was omitted and 2 mM EGTA was added.

Reagents and Stock Solutions

The chemicals, all analytical grade, were obtained from Sigma Chemical (St. Louis, MO) unless otherwise indicated. Fura-2-AM was purchased from Molecular Probes (Eugene, OR). Leukotriene B_4, D_4, C_4 and E_4 were obtained from Cascade Biochem Ltd. (Berkshire, UK). U73122, U73343, and SK&F 96365 were from Biomol Research Lab (PA). Thapsigargin was from Alamone Labs (Jerusalem, Israel). Verapamil was purchased from Research Biochemicals (MA). [2-^{3}H]*myo*-inositol (code TRK 911) was obtained from Amersham International (Buckinghamshire, UK). Ultima Gold was from Packard (Downes Grove, IL).

Fura-2-AM was prepared as a 1 mM stock solution in dry DMSO. The leukotrienes (100 μM) and thapsigargin (1 mM) were dissolved in 96% ethanol and stored under nitrogen. Bradykinin (1 mM), thrombin (1000 IU/ml), gadolinium (10 μM) and verapamil (10 mM) were kept as aqueous stock solutions. All these reagents were stored at (−20°C) until use. U73122 and U73343 (both 10 mM), kept as stock solutions in chloroform, were evaporated and resuspended in 96% ethanol prior to the experiment. SK&F 96365 (10 mM) was prepared as an aqueous solution.

Estimation of $[Ca^{2+}]_i$ Using Fura-2

Loading of Ehrlich cells with fura-2-AM, measurement of $[Ca^{2+}]_i$ and calibration of the fluorescence signal was conducted as described by Jørgensen et al. (1996). Briefly, we proceeded as follows:

Loading of Cells with Fura-2-AM

Cell suspensions (cytocrit 0.4%) were incubated with 2 μM fura-2-AM (standard medium, 0.2% BSA) for 20 min at 37°C. The cells were washed twice and finally resuspended in the experimental buffer to a cytocrit of 5%. When viewed in a fluorescence microscope, Ehrlich cells loaded with fura-2 demonstrated a bright, uniformly distributed fluorescence.

Measurements of $[Ca^{2+}]_i$ in Cell Suspensions

The fura-2 loaded cells were diluted to a cytocrit of 0.5% in the cuvette, and the subsequent fluorescence measurements were conducted in a Perkin Elmer LS-5 Luminescence Spectrometer by continuously shifting the excitation wavelengths between 340 nm and 380 nm under computer control, and collecting emission light at 510 nm.

Calculation of the Intracellular, Free Ca^{2+} Concentration

The fluorescence signal was calibrated by *in vitro* calibration. The intracellular concentration of free calcium, $[Ca^{2+}]_i$, was calculated from the obtained fluorescence ratios according to the equation:

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

where K_d is the dissociation constant (224 nM; Grynkiewicz et al., 1985), R is the fluorescence ratio obtained upon excitation at 340 nm and 380 nm, respectively, and R_{min} and R_{max} are the fluorescence ratios of fura-2 measured in calibration buffers containing either saturating Ca^{2+} or zero Ca^{2+} (2 mM EGTA). S_{f380} and S_{f380} are proportionality coefficients determined by measuring the fluorescence intensity upon excitation at 380 nm using calibration buffers containing zero or saturating Ca^{2+}, respectively (Grynkiewicz et al., 1985).

The values for R_{min}, R_{max} and S_{f380}/S_{b380} were estimated at 0.8, 20.6 and 9.3, respectively, for the experiments presented in Figs. 1, 2, 3. For the data presented in Figs. 4 and 6 another light source was used, and the calibration values thus estimated at 1.36, 111.4 and 31.2, respectively. The excitation spectra of fura-2-P in saturating or zero Ca^{2+} calibration buffers were compared to those of ''cellular'' fura-2, obtained by lysing fura-2-AM loaded cells with digitonin (50 μg/ml), centrifugating, and measuring on the supernatant. The spectra were found to be similar and thus indicating that *in vitro* calibration could be applied.

Estimation of the Cellular Content of Inositolphosphates

Cell suspensions

Six days after intraperitoneal transplantation of Ehrlich cells, the proliferating cells were exposed to 300 μCi of [2-^{3}H]*myo*-inositol using abdominal wall puncture. 18 hrs later, the cells were harvested in low Ca^{2+} medium, sedimented, and subsequently washed twice in low Ca^{2+} medium enriched with 1% BSA (fraction V; dialyzed against water). The cells were then resuspended at a cytocrit of 5% in standard medium containing 1% BSA and incubated 20 min before initiation of the experiment.

Extraction of inositolphosphates

This procedure is essentially as described by N.K. Jørgensen et al. (*submitted*), briefly: 0.5 ml samples of the cell suspension were transferred to Microfuge tubes containing 0.5 ml ice cold PCA (10%) with 0.2% Triton X-100. The samples were subsequently kept on ice for 20 min. During this period, frequent mixing of the samples took place. The tubes were centrifuged (15.000 × *g*, 5 min, 4°C), whereupon 800 μl of the PCA extract was transferred to Microfuge tubes containing 200 μl 10 mM EDTA. The mixture was neutralized with 600 μl of (1:1, v/v) tri-n-octylamine/Freon, as described by Sharpes and McCarl (1982). 800 μl portions of the upper phase were stored in Microfuge tubes at −80°C for subsequent separation of inositolphosphates using the HPLC technique. Samples of the media were treated as the cell samples for analyzing the content of inositolphosphates in the medium. The Ins(1,4,5)P_3 content in the media amounts to 40% of the total Ins(1,4,5)P_3 content in the cell suspension. The cellular content of Ins(1,4,5)P_3 was calculated as described by N.K. Jørgensen et al. (*Submitted*).

Chromatographic Separation of Inositolphosphates

The inositol phosphates were separated by high-performance liquid chromatography (HPLC) using a modified method of Dean and Beaven (1989). We used an anion exchange column (Partisil 5 SAX, RACII, 100 × 4.6 mm from Whatman, New Jersey) equipped with a guard column. The column was equilibrated with 0.01 M ammoniumdihydrogenphosphate buffer, pH 3.8, and eluated with increasing concentrations of the same buffer. The gradient break points given as run time (min) over concentration of eluating buffer (M), were 0/0.01, 5/0.01, 10/0.25, 13/0.28, 15/0.47, 28/0.53, 33/1.4, 45/1.4, 49/0.1. The gradient was tested with adenosine phosphate markers 2–3 times each day by following elution of the markers by their absorption at 254 nm. This gradient separated especially the isomers of inositol triphosphate which eluted after adenosine triphosphate. The effluent was collected and counted in a Liquid Scintillation Counter (Packard).

Abbreviations

DMSO: dimethylsulfoxide; EGTA: ethylene-glycol-bis-β-amino-ethyl-ether)N,N,N′,N′-tetraacetic acid; EDTA: ethylenediaminetetraacetic acid; BSA: Bovine serum albumin; PCA: perchloric acid; AM: acetoxymethyl ester; MOPS: 3-(N-morpholino)propanesulfonic acid; TES: N-tris-(hydroxymethyl)methyl-2-aminoethane sulfonic acid. HEPES: N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; LTB_4, LTC_4, LTD_4 and LTE_4: Leukotriene C_4, D_4 and E_4; PLC: phospholipase C; Ins(1,4,5)P_3: inositol-1,4,5-trisphosphate; DAG: diacylglycerol; U73122: 1-(6-((17β-3-methoxyestra-1,3,5(10)-triene-17-yl)amino) hexyl)-2,5-pyrrolidine-dione; U73343: 1-(6-(17β-3-Methoxyestra-1,3,5(10)-trien-17-yl)×amino)hexyl)-2,5-Pyrrolidine-Dione; SK&F 96365: 1-[β-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl]-1H-imidazole · HCl.

Statistical Evaluation

The values are presented as the mean ± SEM, with the number of independent experiments indicated. Statistical significance was evaluated with a Student's *t*-test, and a $P < 0.05$ was accepted as statistically significant.

Results

LTD_4-induced Ca^{2+} Mobilization

Figure 1 demonstrates that neither LTB_4 (*A*), nor LTC_4 (*B*) or LTE_4 (*C*) are able to mobilize Ca^{2+} or to prevent

Table. Characteristics of the LTD_4-induced Ca^{2+} mobilization

	LTD_4-induced increase in $[Ca^{2+}]_i$		
	Total	Release from internal stores	Influx
100 nM LTD_4	260 ± 14 nM (4)	97 ± 9 nM (4)	114 ± 15 nM (3)[a]
200 nM LTD_4	247 ± 34 nM (4)	104 ± 7 nM (6)	
Maximal value (fitted)	271 nM (4)	115 nM (4)	159 nM (4)[b]
EC_{50}	10 nM (4)	19 nM (4)	6 nM (4)[c]
Sensitive to		U73122 Thapsigargin	U73122
Insensitive to			Verapamil Gadolinium SK&F 96365

Cells were treated as described in the legend to Fig. 1. U73122 (10 μM), thapsigargin (2 μM), verapamil (30 μM), gadolinium (10 μM) and SK&F 96365 (20 μM) were added to block PLC, ER-Ca^{2+}-ATPases, voltage-gated Ca^{2+} (VOC) channels, nonselective stretch activated cation (SA-cat) channels permeable to Ca^{2+}, and receptor operated Ca^{2+} (ROC) channels, respectively. The number of experiments is indicated in brackets. *Maximal value* is the maximal LTD_4-inducible increase in $[Ca^{2+}]_i$, $\Delta[Ca^{2+}]_{i,max}$, estimated from the concentration-response curves in Fig. 2*B*, in which the data were fitted to the Michaelis-Menten equation: $\Delta[Ca^{2+}]_i = (\Delta[Ca^{2+}]_{i,max} + [LTD_4])/(EC_{50} + [LTD_4])$, where $\Delta[Ca^{2+}]_{i,max}$ is the maximal LTD_4-inducible increase in $[Ca^{2+}]_i$, $[LTD_4]$ is the LTD_4 concentration and EC_{50} is the $[LTD_4]$ needed in order to obtain half $\Delta[Ca^{2+}]_{i,max}$.

[a] The size of the $[Ca^{2+}]_i$ peak induced by addition of 100 nM LTD_4 to thapsigargin-treated cells (*see* Fig. 3*B*).

[b] Estimated as the difference between the maximal values for the Total LTD_4-induced increase in $[Ca^{2+}]_i$ and the LTD_4-induced Ca^{2+} Release.

[c] The EC_{50} for the LTD_4-induced Ca^{2+} influx was estimated from the Ca^{2+} Influx curve in Fig. 2*B*.

the LTD_4-induced increase in $[Ca^{2+}]_i$ in Ehrlich cells. Thus, the LTD_4-induced mobilization of Ca^{2+} is not mimicked or blocked by other leukotrienes, in agreement with previous findings that the LTD_4-induced effect on the cell volume could not be mimicked by other leukotrienes e.g., LTB_4, LTC_4 and LTE_4 (Lambert, Hoffmann & Christenen, 1987). That LTD_4 mobilizes Ca^{2+} in Ehrlich cells, as previously demonstrated (Jørgensen et al., 1996, *see* Lambert, 1994, and Hoffmann & Dunham, 1995), is confirmed in Figs. 1 and 2*A*, where it is seen that addition of 50–100 nM LTD_4 to cells suspended in standard medium (1 mM Ca^{2+}) induces a transient increase in $[Ca^{2+}]_i$ followed by a late sustained phase of elevated $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was in four separate experiments estimated at 120 ± 14 nM before stimulation and at 166 ± 10 nM during the late sustained elevated phase.

Figure 2*A* demonstrates, in congruence with the previous findings (Jørgensen et al., 1996), that addition of 100 nM LTD_4 also leads to a transient increase in $[Ca^{2+}]_i$ when the Ehrlich cells are suspended in Ca^{2+}-free medium containing 2 mM EGTA. However, the size of the LTD_4-induced peak in $[Ca^{2+}]_i$ is reduced from ca. 270 nM to ca. 115 nM (*see* the Table) and no late sustained phase of elevated $[Ca^{2+}]_i$ is observed in the absence of extracellular Ca^{2+}. Thus, the LTD_4-induced Ca^{2+} mobilization involves an influx of Ca^{2+} from the extracellular compartment as well as a release of Ca^{2+} from intracellular stores. From Fig. 2*B* is seen, that the size of the LTD_4-induced $[Ca^{2+}]_i$ peak (peak size) in Ehrlich cells suspended in standard medium containing 1 mM Ca^{2+} (*Total*), as well as in Ehrlich cells suspended in Ca^{2+}-free medium containing 2 mM EGTA (*Release*), is concentration-dependent within the LTD_4 concentration range 1–100 nM. Stimulation with a LTD_4 concentration larger than 100 nM does not produce any further increase in the $[Ca^{2+}]_i$ peak size (Fig. 2*B*). Fitting the data in Fig. 2*B* to the Michaelis-Menten expression, the maximal size of the LTD_4-induced increase in $[Ca^{2+}]_i$ and the LTD_4 concentration needed to induce half the maximal value (EC_{50}) have been estimated at 271 nM and 10 nM, respectively, in Ehrlich cells suspended in Ca^{2+}-containing standard medium, and at 115 nM and 19 nM, respectively, in Ehrlich cells suspended in Ca^{2+}-free medium (Table). From the Table it is also seen that maximal Ca^{2+} mobilization is obtained at ca. 100 nM LTD_4. To estimate the maximal $[Ca^{2+}]_i$ value and the EC_{50} value for the LTD_4-induced *Influx* of Ca^{2+}, we subtracted the fitted curve for the total LTD_4-induced increase in $[Ca^{2+}]_i$ from the fitted curve for the LTD_4-induced Ca^{2+} release, whereafter the resulting data were fitted to the Michaelis-Menten expression (*see* Fig. 2*B*, broken line). The maximal $[Ca^{2+}]_i$ value and the EC_{50} value were in this case estimated at

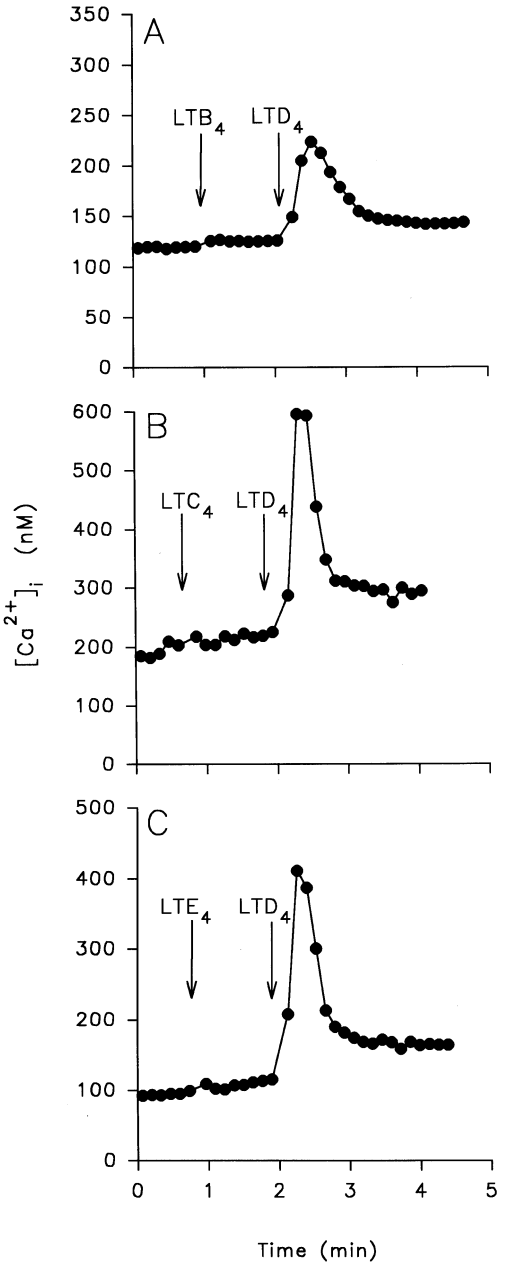

Fig. 1. The effect of LTB_4, LTC_4, LTD_4 and LTE_4 on $[Ca^{2+}]_i$. Ehrlich cells were loaded with fura-2 as described in Materials and Methods, and the cells were incubated in standard medium containing 1 mM Ca^{2+} for 10 to 50 min prior to initiation of the experiment. The experimental cytocrit was 0.5%. $[Ca^{2+}]_i$ was calculated from the ratio of the fluorescence intensities following excitation at 340 nm and 380 nm, respectively, and the emission light was collected at 510 nm. (*A*) The cells were stimulated with 100 nM LTB_4 and 50 nM LTD_4. (*B*) The cells were stimulated with 100 nM LTC_4 and 100 nM LTD_4. (*B*) The cells were stimulated with 100 nM LTE_4 and 100 nM LTD_4. The data presented in Panels *A*, *B* and *C* are representative of three independent sets of experiments.

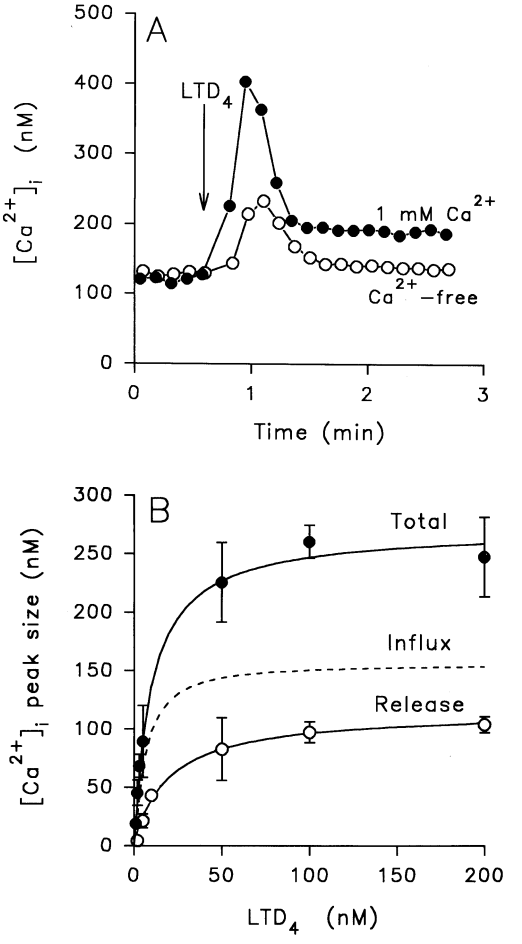

Fig. 2. The effect of external Ca^{2+} on the LTD_4-induced increase in $[Ca^{2+}]_i$, and the concentration-response relationship for the LTD_4-induced increase in $[Ca^{2+}]_i$. Ehrlich cells were loaded with fura-2, incubated for 10 to 50 min in either Ca^{2+}-containing (1 mM Ca^{2+}) or Ca^{2+}-free (2 mM EGTA) medium, and $[Ca^{2+}]_i$ was subsequently estimated as described in the legend to Fig. 1. (*A*) Cells loaded with fura-2 were suspended in either Ca^{2+}-containing medium (1 mM; closed symbols) or Ca^{2+}-free medium (2 mM EGTA; open symbols) and 100 nM LTD_4 was added as indicated by the arrow. (*B*) The $[Ca^{2+}]_i$ peak size, i.e., the maximal $[Ca^{2+}]_i$ value detected after addition of LTD_4, was estimated following addition of LTD_4 in the concentration range 1 nM–200 nM. *Total* (closed symbols) is the $[Ca^{2+}]_i$ peak size estimated in cells suspended in standard medium (1 mM Ca^{2+}). *Release* (open symbols) is the $[Ca^{2+}]_i$ peak size estimated in cells suspended in Ca^{2+}-free medium (2 mM EGTA). The curves (continuous lines) were obtained by fitting the data to the Michaelis-Menten equation. *Influx* (broken line) was calculated as the difference between the ''total'' and the ''release'' curves. Data points represent in each case four-to-eight independent experiments. Similar results with 100 nM LTD_4 have previously been reported in twelve experiments in Ca^{2+}-containing medium and in six experiments in Ca^{2+}-free medium (Jørgensen et al., 1996). The LTD_4 receptor in the Ehrlich cells was previously shown to be desensitized (Jørgensen et al., 1996) and in some recent experiments no detectable increase in $[Ca^{2+}]_i$ was seen after addition of 100 nM LTD_4.

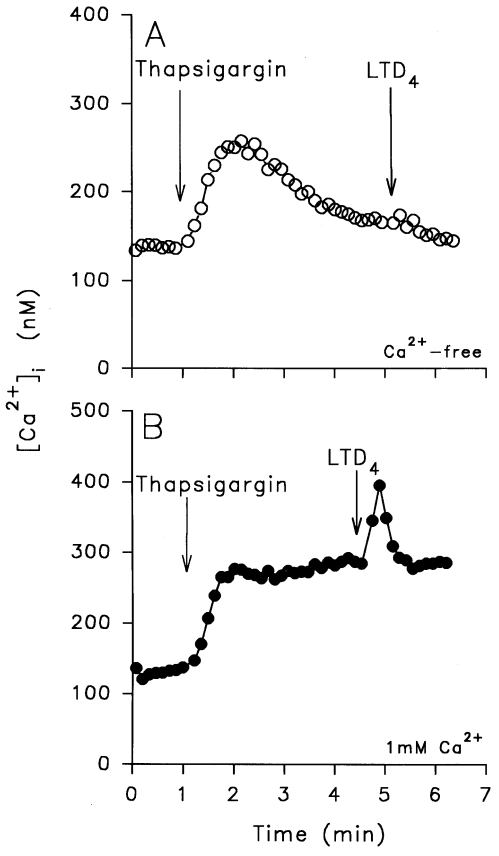

Fig. 3. LTD_4-induced increase in $[Ca^{2+}]_i$ following depletion of the intracellular Ins(1,4,5)P_3-sensitive Ca^{2+} stores with thapsigargin. Ehrlich cells loaded with fura-2 and suspended in either Ca^{2+}-free medium (2 mM EGTA, (*A*) open symbols) or standard medium (1 mM Ca^{2+}, (*B*) closed symbols) were exposed to 2 μM thapsigargin, which blocks ER-Ca^{2+}-ATPases, in order to deplete the intracellular Ins(1,4,5)P_3-sensitive Ca^{2+} stores, and subsequently stimulated with 100 nM LTD_4, as indicated by the arrows. $[Ca^{2+}]_i$ was estimated as described in the legend to Fig. 1. The data shown in *A* and *B* are representative of five and three independent sets of experiments, respectively.

159 nM and 6 nM, respectively, (*see* Table), i.e., at a saturating concentration of LTD_4, Ca^{2+} influx contributes slightly more than Ca^{2+} release to the LTD_4-induced increase in $[Ca^{2+}]_i$.

Figure 3 demonstrates that addition of thapsigargin, an ER-Ca^{2+}-ATPase inhibitor (Thastrup et al., 1990), increases $[Ca^{2+}]_i$ in Ehrlich cells, and it is assumed that the increase in $[Ca^{2+}]$ is due to the depletion of the intracellular Ins(1,4,5)P_3-sensitive Ca^{2+} stores. In the absence of extracellular Ca^{2+}, $[Ca^{2+}]_i$ returns to the initial level within 5 min following addition of thapsigargin (Fig. 3*A*), whereas a sustained elevated $[Ca^{2+}]_i$ is observed in the presence of extracellular Ca^{2+} (Fig. 3*B*). In three paired sets of experiments it has been estimated that addition of 2 μM thapsigargin increases $[Ca^{2+}]_i$ with 111 ± 15 nM within 1 min in Ehrlich cells suspended in standard medium (1 mM Ca^{2+}). Depletion of the intracellular Ca^{2+} stores is known to activate Ca^{2+} channels in the plasma membrane, leading to an inwardly directed Ca^{2+} current (I_{CRAC}, *see* Clapham, 1995). The sustained elevated $[Ca^{2+}]_i$ seen in Fig. 3*B* could, therefore, reflect the presence of depletion operated Ca^{2+} (DOC) channels in Ehrlich cells.

From Fig. 3*A* it is also seen that 100 nM LTD_4 has no effect on $[Ca^{2+}]_i$ in thapsigargin-treated Ehrlich cells suspended in Ca^{2+}-free medium, indicating that LTD_4 in Ehrlich cells mobilizes Ca^{2+} from the intracellular Ins(1,4,5)P_3-sensitive Ca^{2+} stores. On the other hand, from Fig. 3*B* it is seen, that 100 nM LTD_4 induces a transient increase in $[Ca^{2+}]_i$ in thapsigargin-treated Ehrlich cells when these are suspended in standard medium containing 1 mM Ca^{2+}. Thus, the LTD_4-induced increase in $[Ca^{2+}]_i$, seen in thapsigargin-treated Ehrlich cells suspended in Ca^{2+}-containing medium, can be taken to represent the influx of Ca^{2+} from the extracellular compartment. The peak level for the LTD_4-induced Ca^{2+} influx in the thapsigargin treated Ehrlich cells, estimated as the $[Ca^{2+}]_i$ peak size following addition of a saturating portion of LTD_4 (100 nM), has been estimated at 114 ± 15 nM (Table). This peak level, as estimated in thapsigargin-treated Ehrlich cells, is somewhat lower than the peak level, estimated indirectly as the difference between the LTD_4-induced increase in $[Ca^{2+}]_i$ in cells suspended in Ca^{2+}-containing medium and the LTD_4-induced increase in $[Ca^{2+}]_i$ in cells suspended in Ca^{2+}-free medium (Table), most probably because the inwardly directed Ca^{2+} gradient is reduced in the thapsigargin-treated cells.

The exact peak time for the LTD_4-induced increase in $[Ca^{2+}]_i$, i.e., the time between addition of LTD_4 and the highest recorded $[Ca^{2+}]_i$ value is difficult to estimate, because we have a poor resolution in terms of time (approx. 0.14 min between each ratio). Figure 4 shows the actual measured peak times in Ehrlich cells suspended in Ca^{2+}-containing medium (representing Ca^{2+} influx plus Ca^{2+} release from internal stores) or Ca^{2+}-free medium with 2 mM EGTA (representing only the Ca^{2+} release) as well as in cells suspended in Ca^{2+}-containing medium and pretreated with thapsigargin (representing only Ca^{2+} influx). It is seen that after addition of LTD_4 the actual measured $[Ca^{2+}]_i$ peak time resulting from (i) LTD_4-induced Ca^{2+} influx (ii) LTD_4-induced Ca^{2+} release and (iii) LTD_4-induced influx plus the LTD_4 release, is recorded at 0.20 to 0.21 min in four out of five experiments, 0.34 to 0.35 min in six out of eight experiments, and at 0.20 to 0.21 min in ten out of fourteen experiments for the three conditions, respectively. It thus appears that LTD_4 activates the Ca^{2+} influx before the Ca^{2+} release from the internal stores.

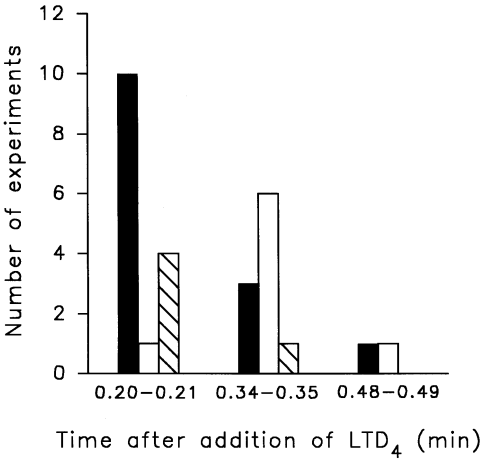

Fig. 4. The peak time for the LTD_4-induced increase in $[Ca^{2+}]_i$. The measured peak time for the LTD_4-induced increase in $[Ca^{2+}]_i$, i.e., the time between addition of 100 nM LTD_4 and detection of the highest measured $[Ca^{2+}]_i$ value, was estimated in Ehrlich cells suspended in (i) standard medium containing 1 mM Ca^{2+} (filled bars, representing both Ca^{2+} influx and Ca^{2+} release from intracellular stores), (ii) Ca^{2+}-free medium containing 2 mM EGTA (open bars, representing Ca^{2+} release) and (iii) standard medium containing 1 mM Ca^{2+} and pretreated with 2 μM thapsigargin (dashed bars, representing Ca^{2+} influx). The number of independent experiments is in each case indicated at the Y-axis.

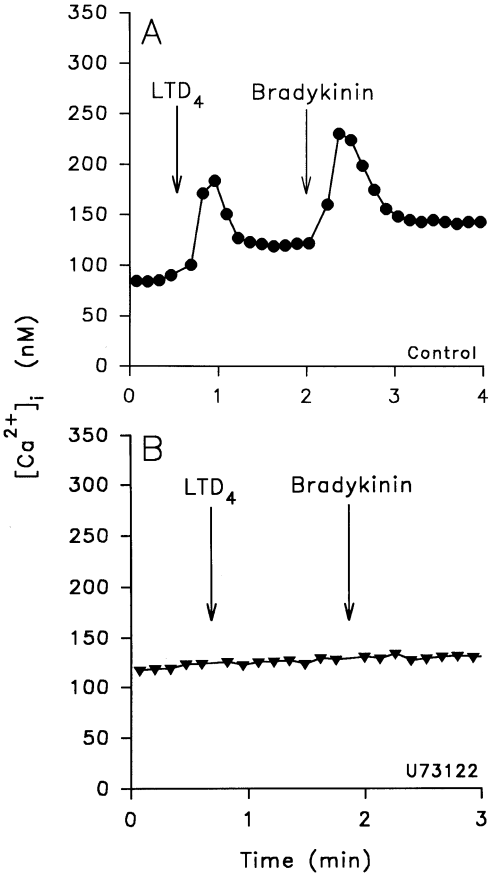

Fig. 5. The effect of the phospholipase C inhibitor U73122 on the LTD_4- and the bradykinin-induced increases in $[Ca^{2+}]_i$. Fura-2 loaded Ehrlich cells were suspended in standard medium (1 mM Ca^{2+}) and $[Ca^{2+}]_i$ measured as described in the legend to Fig. 1. 100 nM LTD_4 and 10 μM bradykinin were added as indicated by the arrows. (*A*) Control cells were preincubated for 2 min with U73343 (10 μM), which is an inactive analogue to U73122 (closed circles). The increases in $[Ca^{2+}]_i$ induced by LTD_4 and bradykinin in the presence of U73343 do not differ from the agonist-induced increases in $[Ca^{2+}]_i$ in control cells from the same day. (*B*) The cells were preincubated 2 min with the PLC inhibitor U73122 (10 μM; closed triangles). The traces shown in *A* and *B* are representative of three and five sets of independent experiments, respectively.

The Role of Phospholipase C and Ins(1,4,5)P_3 in LTD_4-induced Ca^{2+} Mobilization

It is well known, that many agonists inducing receptor-mediated Ca^{2+} mobilization, e.g., bradykinin, often act via an activation of a phospholipase C (PLC) leading to inositoltrisphosphate (Ins(1,4,5)P_3) and diacylglycerol (DAG) formation (*see* Berridge, 1993). The data shown in Fig. 3*B* demonstrates that LTD_4 mobilizes Ca^{2+} from intracellular thapsigargin sensitive Ca^{2+} stores, which in Ehrlich cells has been found to be identical to the intracellular Ins(1,4,5)P_3-sensitive Ca^{2+} stores (Gamberucci et al., 1995), indicating that PLC is involved in the LTD_4-induced Ca^{2+} mobilization in the Ehrlich cells. Figure 5 demonstrates that the effect of LTD_4 on $[Ca^{2+}]_i$ in Ehrlich cells is abolished in the presence of U73122 (*B*), an inhibitor of PLC-dependent processes in polymorphonuclear neutrophils (Smith et al., 1990), whereas U73343 (*A*), the inactive analogue to U73122 (Smith et al., 1990), does not affect neither the LTD_4- nor the bradykinin-induced Ca^{2+} mobilization in Ehrlich cells. Bradykinin has previously been shown to induce a transient increase in the Ins(1,4,5)P_3 content and in $[Ca^{2+}]_i$ in Ehrlich cells (Simonsen et al., 1990). It is noted that U73122 in itself has no detectable effect on $[Ca^{2+}]_i$ (*data not shown*).

Figure 6 shows that LTD_4 produces a transient increase in the Ins(1,4,5)P_3 content in Ehrlich cells, which is reduced in the presence of 100 μM U73122. The Ins(1,4,5)P_3 experiments were performed in the presence of 1% BSA and at a ten times higher cytocrit (5%) than the $[Ca^{2+}]_i$ measurements, which explains why we used 100 μM U73122 compared to the 10 μM used in the Ca^{2+} measurements where no BSA was present. The data in Figs. 5 and 6 indicate that a U73122-sensitive PLC is involved in the LTD_4-induced Ins(1,4,5)P_3 formation as well as in the LTD_4-induced Ca^{2+} mobilization in Ehrlich cells. The observation that U73122 inhibits the LTD_4-induced increase in $[Ca^{2+}]_i$ in Ca^{2+}-containing medium (*see* Fig. 5) seems to indicate an inhibition of the

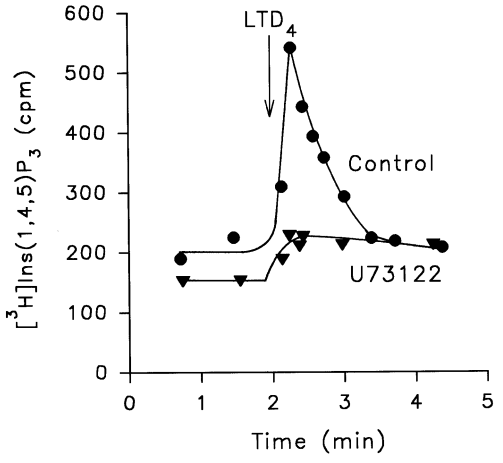

Fig. 6. The effect of LTD_4 on the intracellular Ins(1,4,5)P_3 level. Ehrlich cells labelled *in vivo* for 18 hrs with [2-^{3}H]*myo*-inositol were suspended in standard medium containing 1% BSA. The final cytocrit was 5%. U73122 (100 μM; closed triangles) was added in order to inhibit PLC. Control cells were not treated with U73122 (closed circles). The cells were stimulated with 100 nM LTD_4 as indicated by the arrow. [^{3}H]-inositol phosphates were extracted and separated on an anion exchange column (*see* Materials and Methods). The Ins(1,4,5)P_3 values (shown as cpm) were corrected for a constant background level of extracellular Ins(1,4,5)P_3. The data shown are representative of three independent sets of experiments.

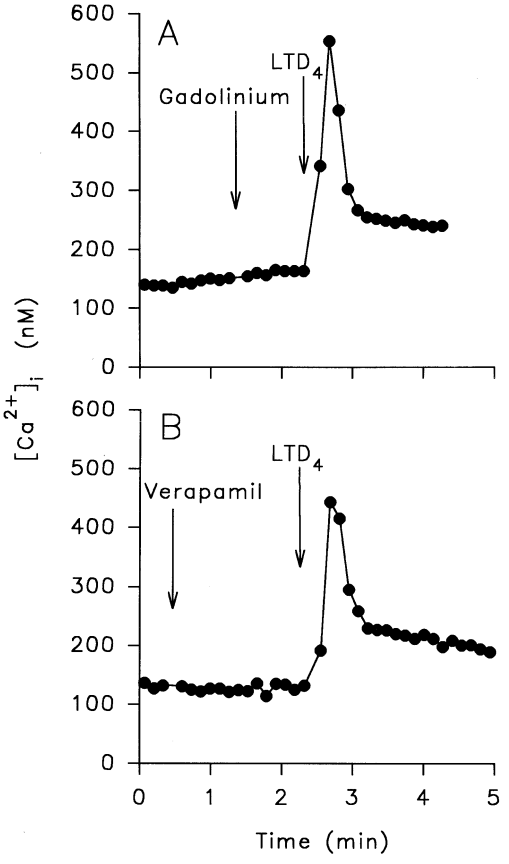

Fig. 7. Effect of gadolinium and verapamil on LTD_4-induced increase in $[Ca^{2+}]_i$. Ehrlich cells were loaded with fura-2 and $[Ca^{2+}]_i$ was estimated as described in the legend to Fig. 1. Gadolinium (10 μM; (*A*)) or verapamil (30 μM; (*B*)) were added as indicated by the arrow to inhibit nonselective stretch-activated cation (SA-CAT) channels and voltage-gated Ca^{2+} (VOC) channels (L-type), respectively. 100 nm LTD_4 was added as indicated by the arrow. The traces in *A* and *B* are representative of three and two independent experiments, respectively. In an additional experiment the LTD_4-induced increase in $[Ca^{2+}]_i$ after stimulation with 50 nM LTD_4 was unaffected by verapamil (*data not shown*).

Ca^{2+} influx. The time between the addition of LTD_4 (100 nM) and the Ins(1,4,5)P_3 peak has been recorded within the time interval 0.27 to 0.30 min ($n = 3$) after addition of LTD_4, i.e., the LTD_4-induced Ins(1,4,5)P_3 peak seems to appear after the LTD_4-induced influx but before the LTD_4-induced release from internal stores (*see* Fig. 4). In a single experiment in Ca^{2+}-free medium, the Ins(1,4,5)P_3 peak time was within the same range (about 0.22 min).

The LTD_4-induced Ca^{2+} Influx

When the intracellular Ins(1,4,5)P_3-sensitive Ca^{2+} stores in Ehrlich cells are depleted by thapsigargin, LTD_4 is no longer able to mobilize any Ca^{2+} from intracellular stores (*see* Fig. 3*A*). The transient increase in $[Ca^{2+}]_i$ following addition of LTD_4 to thapsigargin-treated Ehrlich cells suspended in Ca^{2+}-containing standard medium is, accordingly, taken to indicate that LTD_4 induces a Ca^{2+} influx in the Ehrlich cells. Furthermore, it is assumed that the late elevated $[Ca^{2+}]_i$, seen in Ehrlich cells suspended in Ca^{2+}-containing medium after stimulation with LTD_4 (*see* e.g., Figs. 1 and 2*A*), is due to a sustained influx of Ca^{2+} from the extracellular compartment via the LTD_4-activated Ca^{2+} transport pathways. The experiments shown in Figs. 7 and 8 were initiated to investigate whether the LTD_4-induced Ca^{2+} influx could be via non-selective stretch activated cation (SA-cat) channels, which have been demonstrated in Ehrlich cells by Christensen and Hoffmann (1992), voltage gated Ca^{2+} (VOC) channels or receptor activated Ca^{2+} (ROC) channels. Exposing Ehrlich cells, suspended in Ca^{2+}-containing medium (1 mM Ca^{2+}), to verapamil (30 μM, Fig. 7*A*), which blocks the L-type, VOC channels (Scharff & Foder, 1984), or to gadolinium (10 μM, Fig. 7*B*), which inhibits SA-cat channels permeable to Ca^{2+} (Bennett, 1985), has no effect on the LTD_4-induced increase in $[Ca^{2+}]_i$. Furthermore, the late sustained phase with the elevated $[Ca^{2+}]_i$ seen after LTD_4 addition is not affected by gadolinium or verapamil (Fig. 7). Thus, the LTD_4-induced Ca^{2+} influx seems not to occur via VOC or SA-cat channels (Table).

Depleting the intracellular Ins(1,4,5)P_3-sensitive Ca^{2+} stores with thapsigargin and preincubating the Ehrlich cells with SK&F 96365 (20 μM, 1½ to 2 min),

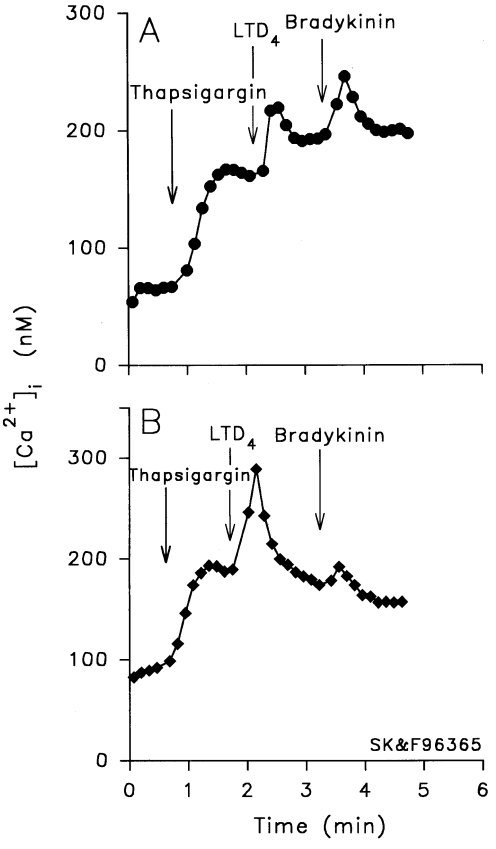

Fig. 8. Effect of SK&F 96365, an inhibitor of receptor operated Ca^{2+} channels, on LTD_4- and bradykinin-induced increase in $[Ca^{2+}]_i$. Ehrlich cells were loaded with fura-2, and the intracellular $Ins(1,4,5)P_3$ sensitive Ca^{2+} stores depleted with thapsigargin, as described in the legend to Fig. 3. 100 nM LTD_4 and 10 μM bradykinin were added as indicated by the arrows. (*A*) Control cells with no further additions (closed circles). (*B*) The cells were preincubated 1½ to 2 min with 20 μM SK&F 96365 to inhibit receptor operated Ca^{2+} (ROC) channels (closed diamonds). The traces in *A* and *B* are representative of three and four independent experiments, respectively.

which blocks ROC channels in human platelets (Merritt et al., 1989), was also without effect on the subsequent LTD_4-induced influx of Ca^{2+} in Ehrlich cells suspended in standard medium containing 1 mM Ca^{2+} (Fig. 8*B*), i.e., the LTD_4-induced Ca^{2+} influx does most probably not occur via ROC channels (Table). On the other hand, the Ca^{2+} influx induced by addition of bradykinin to thapsigargin-treated Ehrlich cells (Fig. 8*A*) seems to be almost absent in the presence of SK&F 96365 (Fig. 8*B*), indicating that a channel of the ROC type is present in the Ehrlich cells.

Discussion

The LTD_4-receptor Coupled to Ca^{2+} Mobilization

It has previously been shown that $[Ca^{2+}]_i$ in Ehrlich cells is transiently increased following addition of LTD_4 (Jørgensen et al., 1996; *see* Lambert, 1994, and Hoffmann & Dunham, 1995). From Figs. 1, 2*A*, 4 and the Table it is seen that the free, intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in Ehrlich cells suspended in Ca^{2+}-containing standard medium increases about 270 nM (peak level) within 0.20 to 0.21 min after addition of 100 nM LTD_4 in ten out of fourteen experiments (peak time), whereafter $[Ca^{2+}]_i$ returns to a sustained level which is elevated compared to the initial level. A LTD_4-induced increase in $[Ca^{2+}]_i$ has also been demonstrated in rat basophilic leukemia cells, RBL-1 (Sarau et al., 1987), in dimethyl sulfoxide-differentiated HL-60 cells (Baud, Goetzl & Koo, 1987), in sheep tracheal smooth muscle cells (Mong et al., 1988), in the human monocytic leukemia cell line, U-937 (Saussay et al., 1989), in the human intestine cell line, Int 407 (Sjölander et al., 1990), and in the human monocytic leukemia cells, THP-1 (Chan et al., 1994). The effects of LTD_4 on $[Ca^{2+}]_i$ in Ehrlich cells is inhibited by the LTD_4 receptor antagonist L-649,923 (Jørgensen et al., 1996), and, furthermore, we demonstrate that neither LTC_4, which is precursor for LTD_4, nor LTE_4, which is the metabolite of LTD_4, or LTB_4 are able to mobilize Ca^{2+} or to block the LTD_4-induced increase in $[Ca^{2+}]_i$ in Ehrlich cells (Fig. 1). Thus, the LTD_4-induced Ca^{2+} mobilization in Ehrlich cells seems to involve a LTD_4 receptor of high specificity. This is at variance with the LTD_4 receptor in the sheep tracheal smooth muscle cells (Mong et al., 1988) and in the differentiated U-937 cell line (Saussy et al., 1989), in which LTE_4 is reported to act as a partial agonist to the LTD_4 receptor. It is noted that LTD_4 in Ehrlich cells also induces a net loss of KCl and cell water, resulting in a significant cell shrinkage (Lambert, 1987, 1989; Lambert et al., 1987). The receptor involved in the LTD_4-induced KCl loss is also sensitive to L-649,923 (Lambert, 1989) and specific for LTD_4 (Lambert et al., 1987). However, LTD_4 seems in the Ehrlich cells to be able to activate the K^+ and Cl^- transporting systems without any measurable increase in $[Ca^{2+}]_i$ (Jørgensen et al., 1996), indicating that the LTD_4 receptor and signaling system coupled to the Ca^{2+} mobilization could be different from the LTD_4 receptor and signaling system coupled to the KCl transporting systems in Ehrlich cells.

LTD_4-induced Ca^{2+} Release from Intracellular Stores Involves PLC activation and $Ins(1,4,5)P_3$ Formation

From Fig. 6 it is seen that LTD_4 induces a transient increase in the intracellular $Ins(1,4,5)P_3$ level in Ehrlich cells. The time between addition of LTD_4 and the $Ins(1,4,5)P_3$ peak (peak time) is estimated to be in the range 0.27 to 0.30 min in three independent sets of experiments (*see* Results), whereas the peak time for the LTD_4-induced Ca^{2+} release, seen in Ehrlich cells sus-

pended in Ca^{2+}-free medium, is in the range 0.34 to 0.35 min in six out of eight independent experiments (Fig. 4). No LTD_4-induced increase in $[Ca^{2+}]_i$ can be observed in Ehrlich cells suspended in Ca^{2+}-free medium and subsequently treated with thapsigargin (Fig. 3*A*). Since the LTD_4-induced increase in $Ins(1,4,5)P_3$ content appears shortly before the LTD_4-induced release of Ca^{2+}, and since both the LTD_4-induced increase in $Ins(1,4,5)P_3$ and the LTD_4-induced increase in $[Ca^{2+}]_i$ are inhibited in the presence of the PLC inhibitor U73122 (Figs. 5 and 6), it is suggested that the LTD_4-induced Ca^{2+} mobilization from intracellular stores in Ehrlich cells involves activation of a U73122-sensitive PLC, $Ins(1,4,5)P_3$ formation and a subsequent release of Ca^{2+} from the $Ins(1,4,5)P_3$-sensitive stores. Grierson & Meldolesi (1995) have reported several unspecific effects of U73122 on the Ca^{2+} homeostasis in mouse fibroblasts. However, according to Berven and Barritt (1995) some of the unspecific effects of U73122 are shared by the inactive analogue U73343. Since we see no effect of U73122 on $[Ca^{2+}]_i$ in Ehrlich cells, and since U73343 does not inhibit the LTD_4-induced mobilization of Ca^{2+}, we find it most likely that the effect of U73122 on the LTD_4-induced Ca^{2+} mobilization reflects an inhibition of PLC. This is in agreement with the observations that LTD_4 stimulation leads to PLC activation in the human intestine cell line, Int 407 (Grönoos et al., 1995), and to phosphatidyl-$Ins(4,5)P_2$ hydrolysis and inositol phosphate formation in rat basophilic leukemia cells (Sarau et al., 1987), guinea pig lung (Mong et al., 1987), sheep tracheal smooth muscle cells (Mong et al., 1988), rat glomerular mesanglial cells (Badr et al., 1989), differentiated U-937 cells (Saussy et al., 1989) and guinea pig tracheal smooth muscle cells (Howard et al., 1992). A similar stimulation of $Ins(1,4,5)P_3$ formation was previously demonstrated in Ehrlich cells with the agonists bradykinin and thrombin (Simonsen et al., 1990).

LTD_4-induced Activation of Ca^{2+} Channels in the Plasma Membrane

Stimulation with LTD_4 is, in general, thought to lead to activation of a Ca^{2+} channel in the plasma membrane providing a Ca^{2+} influx (*see* Crooke et al., 1989, and Sjölander & Grönroos, 1994), and the LTD_4-induced Ca^{2+} influx appears to occur after the LTD_4-induced Ca^{2+} release (Chan et al. 1994; Saussy et al., 1989). In the present study, it is assumed that the increased peak level as well as the late sustained phase of elevated $[Ca^{2+}]_i$ seen after addition of LTD_4 to Ehrlich cells suspended in Ca^{2+}-containing medium, is due to an LTD_4-induced influx of Ca^{2+} (*see* Fig. 2*A*). It is evident that a putative LTD_4-induced inhibition of the Ca^{2+}-ATPase in the plasma membrane would also cause a late sustained phase of elevated $[Ca^{2+}]_i$. In that case, the sustained phase of elevated $[Ca^{2+}]_i$ would be seen in cells suspended in Ca^{2+}-containing medium as well as in cells suspended in Ca^{2+}-free medium. However, we find no sustained phase of elevated $[Ca^{2+}]_i$ in cells suspended in Ca^{2+}-free medium (*see* Fig. 2*A*), excluding any effects of LTD_4 on the Ca^{2+}-ATPase in the plasma membrane of the Ehrlich cells. The LTD_4-induced increase in $[Ca^{2+}]_i$ seen in Ehrlich cells treated with thapsigargin is also taken to represent an influx of Ca^{2+} from the extracellular compartment (*see* Fig. 3*B*). The peak time for the LTD_4-induced Ca^{2+} influx is in the range 0.20 to 0.21 min after addition of LTD_4 in four out of five experiments, whereas the peak time for the LTD_4-induced Ca^{2+} release is in the time range 0.34 to 0.35 min in six out of eight experiments (Fig. 4). Thus, the peak of the LTD_4-induced Ca^{2+} influx in Ehrlich cells seems to appear before the peak in $[Ca^{2+}]_i$ caused by the LTD_4-induced release of Ca^{2+} from the $Ins(1,4,5)P_3$-sensitive Ca^{2+} stores (*see* the Table), which could indicate that LTD_4 activates the Ca^{2+} influx before it releases Ca^{2+} from intracellular stores. It is unlikely that the differences in the $[Ca^{2+}]_i$ peak times reported in the present study are due to the fact that the Ehrlich cells were kept in Ca^{2+}-free medium with EGTA for the measurement of the LTD_4-induced Ca^{2+} release, because we did not record any difference in the peak time for the LTD_4-induced release of $Ins(1,4,5)P_3$ in the absence or in the presence of extracellular Ca^{2+} (*see* results). The present observation that the LTD_4-induced Ca^{2+} influx in the Ehrlich cells seems to appear before the Ca^{2+} release is in contrast to results reported for U-937 cells where estimation of $[Ca^{2+}]_i$ in cell suspensions revealed that the LTD_4-induced increase in $[Ca^{2+}]_i$ is reached faster when the cells are suspended in Ca^{2+}-free medium, i.e., the LTD_4-induced release of Ca^{2+} from intracellular stores comes before the LTD_4-induced Ca^{2+} influx (Saussy et al., 1989).

The apparent EC_{50} for the LTD_4-induced Ca^{2+} *release* and the LTD_4-induced Ca^{2+} *influx* are estimated at 19 nM and 6 nM, respectively (*see* Table), i.e., the LTD_4-induced Ca^{2+} influx in Ehrlich cells occurs at a lower LTD_4 concentration than the LTD_4-induced release of Ca^{2+} from intracellular stores. This is in agreement with the observations made by Jørgensen et al. (1996) that low concentrations of LTD_4 result in an increase in $[Ca^{2+}]_i$ in Ca^{2+}-containing medium (resulting from influx) but not in Ca^{2+}-free medium. The observed difference between the estimated EC_{50} values and between the peak times for the LTD_4-induced Ca^{2+} influx and for the LTD_4-induced Ca^{2+} release could be due to the presence of (i) two LTD_4 receptor subtypes as seen in guinea pig ileum (Gardiner, Abram & Cuthbert, 1990), (ii) one LTD_4 receptor type possessing a low as well as a high affinity binding site as seen in rat glomerular mesanglial cells (Badr et al., 1989) and in the intact rat (Smith et al.,

1989), (iii) one LTD_4 receptor type where the affinity of the receptor towards LTD_4 is reduced upon removal of divalent cations as demonstrated in rat basophilic leukemia cells (Sarau et al., 1987) and THP-1 cells (Rochette, Nicholson & Metters, 1993), or (iv) one LTD_4 receptor type where the function of potentially involved G-proteins is modified due to a change in the transmembrane Ca^{2+} gradient upon removal of external Ca^{2+} as proposed for bovine brain cortices (Fan et al., 1995).

Since the LTD_4-induced peak in $[Ca^{2+}]_i$ due to influx in Ehrlich cells seems to appear before the peak in the $Ins(1,4,5)P_3$ content it is unlikely that opening of the LTD_4-activated Ca^{2+} channel demands $Ins(1,4,5)P_3$ or any other inositolphosphate metabolite. However, the observation that both the LTD_4-induced Ca^{2+} release as well as the LTD_4-induced Ca^{2+} influx are abolished in the presence of the PLC inhibitor U73122 (Fig. 5) could indicate a role of PLC in the LTD_4-induced Ca^{2+} influx in Ehrlich cells. Grönroos et al. (1995) have recently reported that LTD_4-induced Ca^{2+} mobilization in the human intestine cell line, Int 407, involves tyrosine phosphorylation of PLCγ as well as a tyrosine kinase-dependent step, which apparently occurs downstream to PLCγ activation and which is assumed to be implicated in the LTD_4-induced Ca^{2+} influx. In the present study, we are not able to determine whether the LTD_4-induced activation of Ca^{2+} influx in Ehrlich cells involves a tyrosine kinase or whether the effect of the PLC inhibitor U73122 is due to direct or indirect unspecific effects on Ca^{2+} influx as reported by Berven & Barritt (1995) and Grierson & Meldolesi (1995).

Characterization of the LTD_4-activated Ca^{2+} Channel

The exact nature of the LTD_4-activated Ca^{2+} channel in Ehrlich cells remains to be determined. VOC channels are not activated by LTD_4 in dimethylsulfoxide-differentiated HL-60 cells (Baud et al., 1987) or in sheep tracheal smooth muscle cells (Mong et al., 1988). Pertussis toxin is reported to inhibit the LTD_4-induced influx in the U-937 cells (Saussy et al., 1989) and in the human intestine cell line, Int 407 (Sjölander et al., 1990), which has been taken to indicate that the LTD_4 activated Ca^{2+} channel is a G-protein coupled, receptor operated Ca^{2+} channel. The LTD_4-induced increase in $[Ca^{2+}]_i$ in Ehrlich cells as well as the late sustained phase with elevated $[Ca^{2+}]_i$ are not affected by verapamil (Fig. 7*A*) or by gadolinium (Fig. 7*B*), which excludes any LTD_4-induced Ca^{2+} influx via the L-type, VOC channels (Scharff & Foder, 1984) or via SA-cat channels permeable for Ca^{2+} (Bennett, 1985). SA-cat channels, permeable for Ba^{2+} and probably for Ca^{2+}, are well described in Ehrlich cells (Christensen & Hoffman, 1992). Furthermore, no Ca^{2+} channel activation in Ehrlich cells is seen after depolarization from −60 to approximately 0 mV by transferring the cells to high K^+ medium (*data not shown*). Thus, voltage-gated Ca^{2+} channels seem to be absent in the Ehrlich cells. It should be noted, that the only voltage-gated channel reported in Ehrlich cells is the large Cl^- channel (Christensen & Hoffmann, 1992).

Depletion of the intracellular Ca^{2+} stores is known to activate Ca^{2+} channels in the plasma membrane, leading to an inwardly directed Ca^{2+} current via depletion operated Ca^{2+} channels (I_{CRAC}, *see* Clapham, 1995). From Fig. 3*B* it is seen that LTD_4 induces an influx of Ca^{2+} in thapsigargin-treated Ehrlich cells, which can be taken to indicate that the Ca^{2+} channel activated by LTD_4 is not of the depletion operated type. It also seems reasonable to exclude messengers downstream to $Ins(1,4,5)P_3$ in the LTD_4-induced activation of the Ca^{2+} influx, because the $Ins(1,4,5)P_3$ peak seems to appear after the Ca^{2+} influx peak (Fig. 4). SK&F 96365, which is reported to inhibit receptor operated Ca^{2+} channels in human platelets (Merritt et al., 1989), has apparently no effect on the LTD_4-induced Ca^{2+} influx, whereas the bradykinin-induced Ca^{2+} influx is reduced (*see* Fig. 8). This probably reflects that the Ca^{2+} channel activated by LTD_4 and the Ca^{2+} channel activated by bradykinin are distinct. It is noted, that SK&F 96365 is unable to inhibit the ATP-gated Ca^{2+} permeable channel in rabbit ear artery smooth muscle cells and, furthermore, exhibits nonspecific effects (Merritt et al., 1990, Franzius, Hoth & Penner, 1994). Thus, the present observations cannot exclude the involvement of receptor-operated Ca^{2+} channels in the LTD_4-induced Ca^{2+} influx in Ehrlich cells.

In conclusion, LTD_4 acts in the Ehrlich cells via a receptor specific for LTD_4, which upon stimulation provokes (i) an influx of Ca^{2+} through Ca^{2+} channels, which are distinct from Ca^{2+}-depletion operated Ca^{2+} channels, L-type, VOC channels or SA-cat channels, and (ii) to a release of Ca^{2+} from the intracellular $Ins(1,4,5)P_3$-sensitive stores.

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