Leukotriene D_4-induced mobilization of intracellular Ca^{2+} in epithelial cells is critically dependent on activation of the small GTP-binding protein Rho

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We have previously shown that the leukotriene D_4 (LTD_4)-induced mobilization of intracellular Ca^{2+} in epithelial cells is mediated by a G-protein that is distinctly different from the pertussis toxin-sensitive G-protein that regulates the subsequent influx of Ca^{2+}. In the present study, we attempted to gain further knowledge about the mechanisms involved in the LTD_4-induced mobilization of intracellular Ca^{2+} in epithelial cells by investigating the effects of compactin, an inhibitor of the isoprenylation pathway, on this signalling event. In cells preincubated with 10 μM compactin for 48 h, the LTD_4-induced mobilization of intracellular Ca^{2+} was reduced by 75 % in comparison with control cells. This reduction was reversed by co-administration of mevalonate (1 mM). The effect of compactin occurred regardless of whether or not Ca^{2+} was present in the extracellular medium, suggesting that isoprenylation must occur before Ca^{2+} is released from intracellular stores. In accordance with this, we also found that both the LTD_4-induced formation of inositol 1,4,5-trisphosphate and the LTD_4-induced phosphorylation of phospholipase Cγ1 (PLCγ1) on tyrosine residues were significantly reduced in compactin-pretreated cells. These results open up the possibility that the activation of PLCγ1 is related to a molecule that is sensitive to impaired activity of the isoprenylation pathway, such as a small monomeric G-protein. This idea was supported by the observation that *Clostridium botulinum* C3 exoenzyme-induced inhibition of Rho proteins abolished the LTD_4-induced intracellular mobilization of Ca^{2+}. A regulatory role of Rho proteins in the LTD_4-induced activation of PLCγ1 is unlikely to be indirectly mediated via an effect on the cytoskeleton, since cytochalasin D had no major effect on the LTD_4-induced mobilization of Ca^{2+}. Although the mechanism of interaction remains to be elucidated, the present findings indicate an important role of an isoprenylated protein such as Rho in the LTD_4-induced Ca^{2+} signal.

INTRODUCTION

The inflammatory reaction is regulated by a series of complex cell–cell interactions that involve both inflammatory and non-inflammatory cells, and these cell–cell communications are, to a large extent, controlled by soluble inflammatory mediators such as leukotrienes. Different types of leukotrienes have been indicated as crucial mediators in certain inflammatory states, as well as in asthma and hypersensitivity reactions [1,2]. Leukotrienes C_4, D_4 (LTD_4) and E_4, the constituents of the slow-reacting substance of anaphylaxis, are extremely potent and have a large number of physiological effects [1,3]. These effects are generally agreed to be mediated via the interaction of the leukotrienes with specific plasma membrane receptors on the affected cells [4–7].

The regulation of different cellular functions by a variety of extracellular signalling molecules, such as leukotrienes, involves the activation of transmembrane signalling cascades. Most receptor types can be divided into two major groups, based on their intracellular signalling systems. In one of these groups, the receptors are closely related to tyrosine kinase activity, either directly, by means of an endogenous kinase sequence in its cytosolic portion, or indirectly, by an association with a non-receptor tyrosine kinase. In the other group, the receptors initially interact with heterotrimeric G-proteins [8]. In terms of their Ca^{2+} signalling capacities, both types of receptors generally induce activation of a phospholipase C (PLC), although of different isotypes. The tyrosine kinase-linked receptors primarily activate PLCγ, and activated PLCγ is translocated from the cytosol to the plasma membrane and phosphorylated on tyrosine residues. G-protein-coupled receptors, on the other hand, mainly activate PLCβ via interaction with a GTP-bound α subunit and in some cases with a β/γ subunit [8,9]. Regardless of isotype, PLC activation generates an increased accumulation of inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] and, as a direct consequence, mobilization of Ca^{2+} from intracellular stores.

Although no information is available regarding amino acid and nucleotide sequences, the LTD_4 receptor has been characterized as a G-protein-coupled receptor in several types of inflammatory and non-inflammatory cells [7,10]. This is partly due to the findings that the LTD_4 receptor interacts physically with a pertussis toxin-sensitive G-protein [6] and that pertussis toxin impairs certain aspects of the signalling capacity of the LTD_4 receptor in both inflammatory [6,11] and non-inflammatory cells [12]. In human epithelial cells, pertussis toxin abolished only the LTD_4-induced Ca^{2+} influx across the plasma membrane but did not block the G-protein-dependent mobilization of Ca^{2+} from intracellular stores [12]. However, both the mobilization and influx of Ca^{2+} were abolished by guanosine 5′-[β-thio]-diphosphate [12]. In contrast with the general picture of G-protein-coupled receptors, LTD_4-induced Ins(1,4,5)P_3 formation in epithelial cells involves a PLC of the γ1 isotype [13]. In smooth muscle, a similar signalling pathway seems to exist for the angiotensin II receptor (a cloned seven-transmembrane receptor), which also activates PLCγ1 [14]. Considering both the LTD_4 and the angiotensin II receptor, important information is lacking regarding the signalling path-

Abbreviations used: anti-PTyr, anti-phosphotyrosine; Ins(1,4,5)P_3, inositol 1,4,5-trisphosphate; LTD_4, leukotriene D_4; PLC, phospholipase C; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; ECL, enhanced chemiluminescence; GAP, GTPase-activating protein.

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way that leads to the activation of PLCγ1 and the subsequent release of intracellular Ca^{2+}. For the LTD_4-induced mobilization of Ca^{2+}, the significance of a pertussis toxin-insensitive G-protein is of course of special interest [12].

The ability of G-proteins to participate in the transmembrane signalling cascade depends on their position at the inner surface of the plasma membrane. The association of α subunits of heterotrimeric G-proteins with the plasma membrane has been suggested to be due to a post-translational myristoylation [15], whereas the association of β/γ subunits is facilitated by a post-translational isoprenylation at the cysteine in the C-terminal CXXX sequence of the γ subunit [16,17]. In addition, monomeric small G-proteins are also directed to the plasma membrane via an identical isoprenylation [18–20]. The functional roles of protein isoprenylation can be studied experimentally by using specific competitive inhibitors of the isoprenylation pathway, such as compactin or lovastatin (mevinolin) [21]. These compounds inhibit the enzyme 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, which synthesizes mevalonate, a precursor of isoprenoids [22]. The effects of compactin or lovastatin can be tested by co-administering exogenous mevalonate, i.e. such treatment will reverse the effects of compactin or lovastatin, if they are due to impaired protein isoprenylation.

The aim of the present study was to use compactin to gain further knowledge about the involvement of G-proteins in the signalling mechanism of LTD_4-induced mobilization of intracellular Ca^{2+} in epithelial cells.

MATERIAL AND METHODS

Materials

The anti-PLCγ1 antibodies and *Clostridium botulinum* C3 exoenzyme were obtained from Upstate Biotechnology (Lake Placid, NY, U.S.A.), the anti-RhoA and anti-RhoB antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.), and anti-phosphotyrosine (anti-PTyr) antibody (PY20) was from Transduction Laboratories (Lexington, KY, U.S.A.). Normal mouse serum was purchased from Dako A/S (Copenhagen, Denmark), and LTD_4 was from Cayman Chemical Company (Ann Arbor, MI, U.S.A.). The Ins(1,4,5)P_3 assay system, all enhanced chemiliminescence (ECL) Western-blot detection reagents and the Hyperfilm used were from Amersham International (Amersham, Bucks., U.K.); other chemicals, including the D,L-mevalonic acid lactone, were of analytical grade and obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Cell culture

The Intestine 407 cell line, isolated from the jejunum and ileum of a human embryo of about two months' gestation [23], was obtained from Flow Laboratories (Rickmansworth, U.K.). The cells were cultured as a monolayer to confluence for 5 days (in culture flasks or on coverslips) in Eagle's basal medium supplemented with 15% newborn calf serum, 55 IU/ml penicillin and 55 μg/ml streptomycin. The cells were kept at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. The cells were regularly tested for the absence of mycoplasma contamination. In certain experiments (indicated in the legends to the Figures), the cell culture medium was supplemented with 10 μM compactin for the last 48 h.

Electro-permeabilization of cells in culture

The cells were permeabilized by electric discharge as previously described [12,24]. In short, a coverslip with a confluent layer of cells (see below) was placed parallel to the electrical field in a chamber containing a medium (37 °C) with the following composition: 20 mM NaCl, 100 mM KCl, 1 mM NaH_2PO_4, 25 mM $NaHCO_3$, 20 mM Hepes, 1 mM EGTA and 0.2% (w/v) BSA, pH 7.0, with or without a supplement of C3 exoenzyme (4 μg/ml). The cells were rendered permeable by repeated exposures (150 μs each) to an electrical field of 1.7 kV/cm, until approx. 70% of the cells were positively stained with Trypan Blue (5.0 mg/ml). The coverslip was then transferred to a Petri dish containing the basal culture medium, lacking serum and with or without C3 exoenzyme (4 μg/ml), for approx. 1 h at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. No morphological differences were seen between cells electro-permeabilized in the presence and the absence of C3 exoenzyme, and less than 5% of all the permeabilized cells were stained with Trypan Blue after the final 1 h incubation in culture medium.

Determination of cytosolic free Ca^{2+} levels

The cells were cultured for 5 days on a round coverslip (25 mm in diameter) placed on the bottom of a Petri dish (35 mm × 10 mm). For the last 48 h of this period the cells were cultured in the absence or presence of compactin (10 μM). Thereafter, the cells were incubated with fura 2/AM (4 μM) in fresh culture medium (with or without compactin) for 30 min at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. After fura 2 loading, the coverslip was placed in a specially designed chamber in which it constituted the bottom. To this chamber was added 0.5 ml of a calcium-containing medium with the following composition: 136 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 5.0 mM $NaHCO_3$, 20 mM Hepes, 1.0 mM $CaCl_2$ and 5.5 mM glucose (pH 7.4) or 0.5 ml of a calcium-free buffer with the same composition as above except without $CaCl_2$ but supplemented with 1.0 mM EGTA. The concentration of cytosolic free Ca^{2+} was then measured with dual excitation microfluorimetry, essentially as specified by Grynkiewicz and co-workers [25]. In our case, the cytosolic free Ca^{2+} determinations were performed on a NIKON inverted microscope (epifluorescence mode, objective NIKON F100). The excitation alternated rapidly (4 times/s) between $\lambda_1 = 340$ nm and $\lambda_2 = 380$ nm (Spex Fluorometer, Glen Creston, London, U.K.). This set-up was also equipped with a thermostatic chamber enabling the cells to be maintained at 37 °C during the experiment. The fluorescence was monitored through an interference filter ($\lambda = 510$ nm) by photon counting. The ratio of the fluorescence at the two wavelengths was calibrated to express the cytosolic free calcium concentration as previously described [25].

Determination of the cellular content of Ins(1,4,5)P_3

Cells growing in culture flasks were first washed twice in calcium medium and allowed to rest for at least 30 min. After this resting period the cells were incubated with or without 40 nM LTD_4. These stimulations were terminated after the periods of time indicated in Figure 3 by rapidly discarding the incubation medium and adding 3 ml of ice-cold trichloroacetic acid (final concentration 15%, v/v) to stop the reaction. The flasks were then immediately placed on ice, and the cells were scraped loose from the bottom of each flask. The cells were kept on ice for 15 min and then centrifuged. The resulting supernatants were washed three times with a 5-fold excess of diethyl ether, after which the pH was adjusted to 7.5 with 2 mM Tris. Following evaporation, the samples were stored frozen until analysed. The cellular Ins(1,4,5)P_3 content was assayed with a commercial kit (Amersham) based on competitive binding of the metabolite to an Ins(1,4,5)P_3-binding protein.

Immunoprecipitation

The cells were first washed twice with calcium medium and then allowed to rest for 30 min in such medium in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C before being stimulated with 40 nM LTD_4 for 15 s. The immunoprecipitations were performed on membrane and cytosolic fractions obtained essentially as described by Mumby and co-workers [26]. In short, the stimulations were terminated by adding an ice-cold buffer containing 20 mM NaHepes (pH 8.0), 1 mM EDTA and 2 mM $MgCl_2$, supplemented with the following protease inhibitors: 2 mM Na_3VO_4, 60 μg/ml PMSF and 4 μg/ml leupeptin. To ensure satisfactory lysis, the cells were scraped loose into the buffer and homogenized 10 times on ice. Nuclei and cell debris were then cleared by initial centrifugation at 1000 ***g*** for 5 min at 4 °C. The protein contents of the remaining supernatants were then measured and compensated for so that all samples had an equal amount of starting material for the subsequent isolations. Each supernatant was centrifuged at 200000 ***g*** for 30 min at 4 °C to collect membranes and the supernatant (600 μl each) used as cytosol. The membrane fractions were washed once before being resuspended in 600 μl of the buffer described above. The amount of solubilized proteins was determined for each sample and corrections were made so that an equal amount from each sample was subsequently processed. All fractions were then pre-cleared by incubation with 100 μl of Protein A–Sepharose [20 mg/ml; preincubated with 1% (v/v) normal mouse sera] for 1 h at 4 °C and thereafter centrifuged at 10000 ***g*** for 2 min at 4 °C. Immunoprecipitations were then performed on the obtained supernatants by incubating the fractions for 1 h at 4 °C with 100 μl of Protein A–Sepharose (20 mg/ml) pre-incubated (1 h at 4 °C) with anti-PLCγ1 (3.5 μg/ml). The immunoprecipitates were recovered by centrifuging at 10000 ***g*** for 2 min at 4 °C and then washing (three times) with lysis buffer before further processing.

Gel electrophoresis

The immunoprecipitated proteins were solubilized by boiling at 100 °C for 5 min in a sample buffer consisting of 62 mM Tris (pH 6.8), 1.0% SDS, 10% glycerol, 15 mg/ml dithiothreitol and 0.05% Bromophenol Blue. All samples were then subjected to gel electrophoresis in the presence of SDS in 8 or 12% homogeneous polyacrylamide gels [27].

Immunoblotting

The separated proteins were electrophoretically transferred to a poly(vinylidine difluoride) (PVDF) membrane (NEN Dupont, Boston, MA, U.S.A.). After blocking the PVDF membrane with 3% BSA, the blot was incubated with either anti-PLCγ1 (1:1000 dilution), anti-PTyr (1:1000 dilution), anti-RhoA (1:200) or anti-RhoB (1:200) antibodies overnight at 4 °C. The membrane was then washed extensively and incubated with peroxidase-linked goat anti-mouse or goat anti-rabbit antibodies (1:10000 dilution) for 1 h at room temperature. Thereafter, the membrane was washed again, incubated with the Western-blot reagents of a commercial ECL kit from Amersham and exposed to hyperfilm (Amersham) to reveal the immunoreactive proteins. In some experiments, after analysing the initial results, the blots were washed with 100 mM 2-mercaptoethanol, 2% SDS and 62.5 mM Tris/HCl (pH 6.7) for 30 min at 50 °C to remove bound antibodies. The PVDF membrane was then reprobed with a new antibody, as described above. Densitometric analysis was performed on a LKB Ultroscan XL enhanced laser densitometer (Bromma, Sweden).

RESULTS

Effects of compactin on LTD_4-induced Ca^{2+} signalling

The effects of compactin (10 μM for 48 h) alone on resting cytosolic free Ca^{2+} concentrations in single Intestine 407 cells and on cytosolic free Ca^{2+} responses triggered by LTD_4 (40 nM) are outlined in Figure 1. In the presence of extracellular calcium (Figure 1A), no effect on the resting cytosolic free Ca^{2+} concentration was noted, whereas the LTD_4-induced cytosolic free

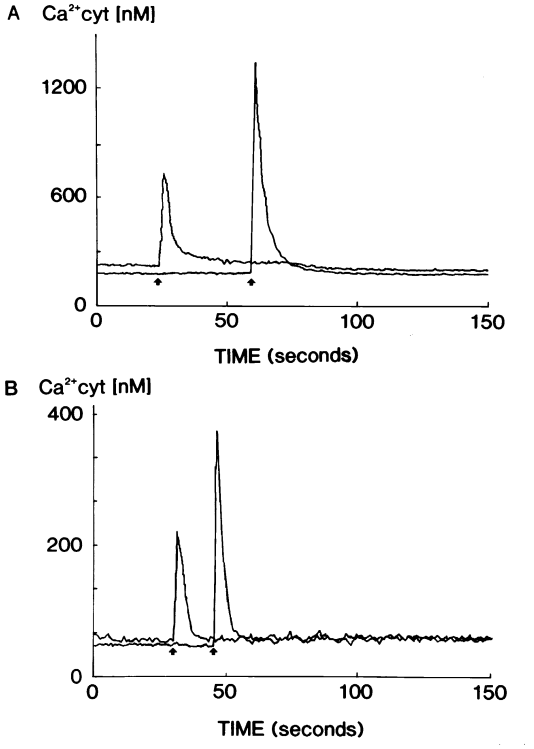

Figure 1 Effects of compactin on the LTD_4-induced cytosolic (cyt) free Ca^{2+} response in single, fura 2-loaded Intestine 407 cells

(**A**) Cytosolic free Ca^{2+} responses of cells stimulated with 40 nM LTD_4 (arrows) in the presence of extracellular calcium (1 mM); (**B**) responses after such stimulations (arrows) in the absence of extracellular calcium (calcium-free medium supplemented with 1 mM EGTA). In both panels, the first and second cytosolic free Ca^{2+} transients respectively correspond to a cell pre-incubated with compactin (10 μM) for 48 h and a cell pre-incubated in the absence of compactin. All traces are representative of at least 10 separate experiments.

Table 1 Statistical analysis of the effects of compactin on the LTD_4-induced cytosolic free Ca^{2+} responses in Intestine 407 cells

Effects of compactin on the LTD_4-induced cytosolic free Ca^{2+} response in Intestine 407 cells (Figure 1) were statistically analysed by measuring the individual peak area (arbitrary units) of each cytosolic free Ca^{2+} transient. The responses are from cells incubated in the absence or the presence of 10 μM compactin for 48 h. The presence or absence of extracellular calcium is indicated in the Table. The values given are means ± S.E.M. from 10–20 separate experiments.

	Integrated peak area (arbitrary units)	
Conditions	Ca^{2+}-depleted medium	Ca^{2+} medium
Control	2.7 ± 0.8	13.0 ± 2.3
Compactin	0.7 ± 0.3	2.8 ± 0.8

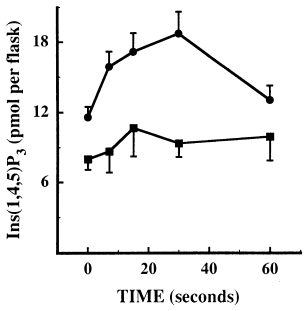

Figure 2 Effects of compactin on the LTD_4-induced formation of Ins(1,4,5)P_3

Effects of LTD_4 stimulation (40 nM) on the cellular content of Ins(1,4,5)P_3 in cells pre-incubated in the absence (●) or the presence (■) of 10 μM compactin for 48 h. The stimulations with LTD_4 were terminated by the rapid addition of ice-cold trichloroacetic acid after the indicated periods of time, and the cellular contents of Ins(1,4,5)P_3 were determined as described in the Materials and methods section. The results outlined in the Figure are means ± S.E.M. from 4–6 separate experiments.

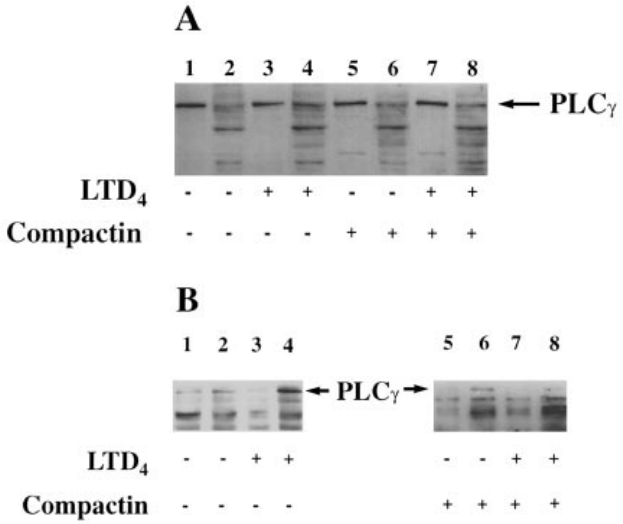

Figure 3 Effects of compactin on LTD_4-induced tyrosine phosphorylation and translocation of PLCγ1

(**A**) Western-blot analysis of immunoprecipitates obtained with an anti-PLCγ1 antibody (3.5 μg/ml) from cytosolic (lanes 1, 3, 5 and 7) and membrane (lanes 2, 4, 6 and 8) fractions of unstimulated cells (lanes 1, 2, 5 and 6) and from cells stimulated for 15 s with 40 nM LTD_4 (lanes 3, 4, 7 and 8). The results shown in lanes 1–4 are from cells pre-incubated without compactin, and the responses in lanes 5–8 are from cells pre-incubated in the presence of 10 μM compactin for 48 h. The blot was developed with an anti-PLCγ1 antibody (1:1000) and revealed with an ECL Western-blot detection kit. The outlined results are representative of five separate experiments. (**B**) Western-blot analysis of immunoprecipitates obtained with an anti-PLCγ1 antibody (3.5 μg/ml) from cytosolic (lanes 1, 3, 5 and 7) and membrane (lanes 2, 4, 6 and 8) fractions of unstimulated cells (lanes 1, 2, 5 and 6) and from cells stimulated for 15 s with 40 nM LTD_4 (lanes 3, 4, 7 and 8). The results shown in lanes 1–4 are from cells pre-incubated without compactin, and the responses in lanes 5–8 are from cells pre-incubated in the presence of 10 μM compactin for 48 h. The blot was developed with an anti-PTyr antibody (1:1000) and revealed with an ECL Western-blot detection kit. The outlined results are representative of five separate experiments.

Ca^{2+} response was significantly reduced after compactin treatment (first transient, Figure 1A) as compared with cells not pretreated with compactin (second transient, Figure 1A). The effect of compactin on LTD_4-induced Ca^{2+} signalling was also tested in the absence of extracellular calcium (Figure 1B). The results shown in Figure 1(B) are similar to those illustrated in Figure 1(A) and show that compactin had no effect on the resting cytosolic free Ca^{2+} concentration but did reduce the LTD_4-induced cytosolic free Ca^{2+} response by approx. 75% (first transient, Figure 1B) as compared with the response of cells not pre-treated with compactin (second transient, Figure 1B). The effects of compactin on the LTD_4-induced cytosolic free Ca^{2+} responses were also analysed in several separate single-cell experiments by integrating the peak area of each single response. The statistical analysis of these data, shown in Table 1, confirms the results of the representative recordings from single epithelial cells (Figures 1A and 1B). All of the above-mentioned effects were reversed (by > 80%) when mevalonate (1 mM) was co-administered with compactin (results not shown). In non-excitable cells, the influx of Ca^{2+} is generally regulated by the release of Ca^{2+} from intracellular stores [28], a mechanism that also appears to apply to the LTD_4-induced influx of Ca^{2+} in the present cell line [13]. This suggests that the present effects of compactin are most likely located upstream of the LTD_4-induced mobilization of intracellular Ca^{2+}.

Effect of compactin on LTD_4-induced Ins(1,4,5)P_3 formation

Considering the results described above, we continued our work by studying the effects of compactin on the cellular formation of Ins(1,4,5)P_3. The results show that pretreatment with compactin (10 μM for 48 h) reduced the resting level of Ins(1,4,5)P_3 and also significantly impaired the LTD_4-induced formation of this Ca^{2+}-mobilizing messenger (Figure 2). The finding that the LTD_4-induced formation of Ins(1,4,5)P_3 in these cells is mediated via an activation of PLCγ1 [13] made us investigate the effects of compactin on the cellular handling of this key enzyme in the Ca^{2+} signalling pathway.

Effects of compactin and LTD_4 on the membrane association and tyrosine phosphorylation of PLCγ1

In accordance with other agonists that have been shown to activate the γ isotype of PLC, stimulation with LTD_4 resulted in a prompt accumulation of PLCγ1 in a membrane fraction derived from Intestine 407 cells (Figure 3A, lane 4). This effect of LTD_4 was not observed in cells that had been pretreated with compactin (Figure 3A, lane 8). Regardless of whether or not the cells had been stimulated with LTD_4, a significant level of PLCγ1 was noted in the membrane fraction of these compactin-pretreated cells (Figure 3A, lanes 6 and 8).

To obtain an indication of the activity of PLCγ1 in the cytosolic and membrane fractions, we performed Western-blot analysis with anti-PTyr antibodies from these anti-PLCγ1 immunoprecipitates (Figure 3B). The results revealed that LTD_4 induced an increase in tyrosine phosphorylation of PLCγ1 in the absence of compactin (Figure 3B, lane 4), an effect that was abolished in compactin-pretreated cells (Figure 3B, lane 8). The LTD_4-induced increase in phosphorylation was 166 ± 16% (n = 5) in non-pretreated cells, whereas it was slightly reduced (72 ± 8%) in compactin-pretreated cells, as quantified by densitometric analysis. Although PLCγ1 was present in the membrane fractions of the compactin-pretreated cells (Figure 3A, lanes 6 and 8), it was only weakly phosphorylated on tyrosine residues (Figure 3B, lanes 6 and 8).

Effects of C3 exoenzyme on LTD_4-induced mobilization of intracellular Ca^{2+}

The involvement of Rho proteins in LTD_4-induced mobilization of Ca^{2+} was tested by using C3 exoenzyme, which specifically

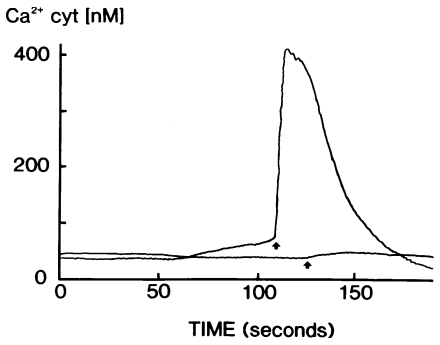

Figure 4 Effects of C3 exoenzyme on the LTD_4-induced mobilization of intracellular Ca^{2+} in single, fura 2-loaded Intestine 407 cells

Cytosolic (cyt) free Ca^{2+} responses of cells stimulated with 40 nM LTD_4 (arrows) in the absence of extracellular calcium (calcium-free medium supplemented with 1 mM EGTA). The traces represent electro-permeabilized cells incubated in either the absence (first arrow) or the presence (second arrow) of 4 μg/ml C3 exoenzyme for a total of 60 min prior to the addition of fura 2, as described in the Materials and methods section. The outlined results are representative of 10 separate experiments.

Table 2 Statistical analysis of the effects of C3 exoenzyme on the LTD_4-induced cytosolic free Ca^{2+} response in Intestine 407 cells

Effects of C3 exoenzyme (4 μg/ml; Figure 4) were further analysed by measuring both the ΔCa^{2+} (nM) and the individual peak area (arbitrary units) of each LTD_4-induced cytosolic free Ca^{2+} transient. The cells were electro-permeabilized in the absence (control) or in the presence of C3 exoenzyme and then stimulated in the absence of extracellular calcium. The values given are means $\pm$ S.E.M. from 10 separate experiments.

Conditions	ΔCa^{2+} (nM)	Integrated peak area (arbitrary units)
Control	163 ± 29	4.7 ± 0.9
C3 exoenzyme	16 ± 7	0.6 ± 0.3

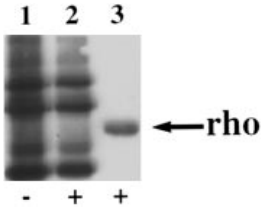

Figure 5 Effects of LTD_4 on the association of Rho GTP-binding proteins with PLCγ1

Western-blot analysis of immunoprecipitates obtained with a PLCγ1 antibody (3.5 μg/ml) from cell lysates (lanes 1 and 2); as a control a whole-cell lysate was also analysed (lane 3). The cells were either unstimulated (lane 1) or stimulated for 15 s with 40 nM LTD_4 (lanes 2 and 3). The blot was developed with an anti-RhoA antibody (1:200) and revealed with an ECL Western-blot detection kit. The outlined results are representative of five separate experiments.

inactivates all Rho proteins by ADP ribosylation and thereby prevents any regulation of their downstream targets [29]. The effects of electro-permeabilization in the absence or presence of C3 exoenzyme (4 μg/ml for 1 h) on the LTD_4 (40 nM) -triggered cytosolic free Ca^{2+} response in a calcium-depleted medium are outlined in Figure 4. As can be seen in Figure 4, neither electro-permeabilization nor the simultaneous addition of C3 exoenzyme

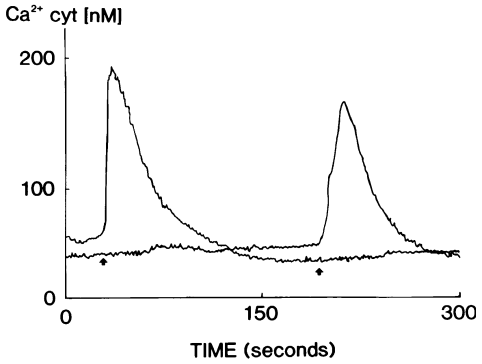

Figure 6 Effects of cytochalasin D on the LTD_4-induced cytosolic (cyt) free Ca^{2+} response in single, fura 2-loaded Intestine 407 cells

Cytosolic free Ca^{2+} level of cells exposed to cytochalasin D (5 μg/ml) or DMSO alone and then stimulated with 40 nM LTD_4 (arrows) in a calcium-free medium supplemented with 1 mM EGTA. The first and second cytosolic free Ca^{2+} transients respectively correspond to a cell pre-incubated with DMSO alone and a cell pre-incubated with cytochalasin D. Both traces are representative of eight separate experiments.

appeared to have any major effect on the resting cytosolic free Ca^{2+} level in these cells. In addition, electro-permeabilization alone only modestly affected the LTD_4-induced (arrow) cytosolic free Ca^{2+} response (Figure 4, first trace) as compared with such a response in non-permeabilized cells (Figure 1B). In contrast, stimulation with LTD_4 (arrow) did not induce a detectable mobilization of intracellular Ca^{2+} from cells electro-permeabilized in the presence of C3 exoenzyme (Figure 4, second trace). This effect of C3 exoenzyme on the LTD_4-induced mobilization of intracellular Ca^{2+} was also confirmed by analysing both the ΔCa^{2+} response and the peak area obtained by integrating the responses recorded in several single cell experiments (Table 2). The statistical analysis of the data (Table 2) confirms the results of the representative recordings from single epithelial cells (Figure 4).

Effect of LTD_4 on the association of PLCγ1 with Rho proteins

A possible molecular interaction between Rho proteins and PLCγ1 was tested by Western-blot detection of Rho proteins in anti-PLCγ1 immunoprecipitates of lysates from unstimulated cells (Figure 5, lane 1) or cells stimulated with 40 nM LTD_4 for 15 s (Figure 5, lane 2). The results presented in Figure 5 (lanes 1 and 2) clearly show that it was impossible to detect any direct association between RhoA proteins and PLCγ1 with the immunoprecipitation approach used in this study, although RhoA proteins were clearly detected in whole cell lysates (Figure 5, lane 3). Similar results were obtained with RhoB antibodies (results not shown). These findings do not exclude the possibility that a weak or a very transient (due to a prompt GTPase activation) association between Rho proteins and PLCγ1 can occur as an important regulatory step in the transduction pathway between the receptor for LTD_4 and the mobilization of intracellular Ca^{2+}.

Effects of cytochalasin D on LTD_4-induced Ca^{2+} signalling

To exclude the possibility that the effects of C3 exoenzyme are indirectly mediated via the well-known effect of Rho proteins on the cellular cytoskeleton [19], we also tested the effects of cytochalasin D (5 μg/ml for 5 min) on the LTD_4 (40 nM) -triggered mobilization of intracellular Ca^{2+} (Figure 6). As can be

seen, addition of cytochalasin D in a Ca^{2+} depleted medium had no major effect on the resting cytosolic free Ca^{2+} level nor on the LTD_4-induced (arrows) cytosolic free Ca^{2+} response (Figure 6, second transient) as compared with such a response in cells exposed to DMSO alone (Figure 6, first transient). The lack of effect of cytochalasin D on the LTD_4-induced mobilization of intracellular Ca^{2+} was confirmed by analysing the ΔCa^{2+} response in each single experiment. This analysis revealed that the ΔCa^{2+} response of cells exposed to DMSO alone was 119 ± 13 nM ($n = 8$) whereas that of cells exposed to cytochalasin D was 100 ± 13 nM ($n = 8$).

DISCUSSION

The present investigation shows that a normally functioning isoprenylation pathway is a prerequisite for LTD_4-triggered formation of Ins(1,4,5)P_3 and the subsequent mobilization of intracellular Ca^{2+} in epithelial cells. The fact that the inhibiting effects of compactin were substantially (by 80 % or more) reversed by the addition of mevalonate, the first committed intermediate of the isoprenoid pathway, indicates that the loss of signalling activity is indeed related to the lack of a pathway intermediate. Deanin and co-workers [30] found that a lovastatin-induced inhibition of the isoprenylation pathway in leucocytes blocked antigen-stimulated production of Ins(1,4,5)P_3 and the subsequent mobilization of intracellular Ca^{2+}, results very similar to the present data on compactin-induced effects on LTD_4-triggered Ca^{2+} signalling. In contrast, it has also been reported that lovastatin pretreatment has no effect on agonist-induced cytosolic free Ca^{2+} signalling in either insulin-producing HIT-T15 cells [31] or N1E-115 neuronal cells [32]. Moreover, Haag and co-workers [33] found that lovastatin pretreatment impaired the agonist-induced Ca^{2+} influx in differentiated HL60 cells by 40–60 %. The latter report is, however, not directly applicable to the present study, since the LTD_4-induced influx of Ca^{2+} appears to be regulated by a pertussis toxin-sensitive heterotrimeric G-protein and protein tyrosine phosphorylations [12,13]. Consequently, inhibition of the isoprenoid pathway must have totally different effects on cytosolic free Ca^{2+} signalling induced by distinctly different receptors in various types of cells. This could be readily explained if miscellaneous and distinct signalling components couple these receptors to the release and influx of Ca^{2+}. The transduction mechanisms by which extracellular molecules such as LTD_4 mobilize intracellular Ca^{2+} via an isoprenylation-dependent pathway must therefore be further characterized to allow identification of the element(s) involved in this signalling system.

In considering the critical role of the isoprenylation pathway in intracellular signal transduction, the majority of the obtained results have particularly focused the attention on modifications and translocations of small monomeric G-proteins. In the present study such an interpretation is also relevant, since we here show that inactivation of a specific group of small monomeric G-proteins, the so-called Rho proteins, by introduction of C3 exoenzyme results in the abolition of the LTD_4-induced mobilization of intracellular Ca^{2+}. Furthermore, we have previously shown that the LTD_4-induced Ca^{2+} signal is mediated via activation of PLCγ1 [13]. The fact that Rho proteins have been proposed as critical regulators of key enzymes of the phospholipid metabolism [34–37] lends additional support for a role of these Ras-related proteins in the regulation of the LTD_4-induced Ca^{2+} release. Considering the immediate onset of the LTD_4-induced release of intracellular Ca^{2+}, it is possible that the active form of Rho proteins in the present study could directly or indirectly participate in the regulation of PLCγ1.

The assumption that PLCγ1 is the enzyme that is regulated by the Rho proteins in this study is based on the findings that compactin severely diminished both the LTD_4-induced tyrosine phosphorylation of PLCγ1, a phenomenon related to the activation of this enzyme, and the LTD_4-induced formation of Ins(1,4,5)P_3. In a different system, however, it was recently suggested that the ability of lovastatin to decrease the mobilization of intracellular Ca^{2+} in response to thrombin and platelet-derived growth factor might be related to the ability of C3 exoenzyme to impair phosphatidylinositol 4-phosphate 5-kinase activity in cell lysates [36]. The idea that enzymes involved in the formation and the availability of phosphatidylinositol 4,5-bisphosphate, the substrate for PLC, could participate in the regulation of agonist-induced mobilization of intracellular Ca^{2+} are interesting. However, the fact that we noted an immediate onset of the LTD_4-induced Ca^{2+} signal in epithelial cells, which is in contrast with the more gradual onset of at least the platelet-derived growth factor-induced Ca^{2+} signal in C3H 10T1/2 cells [36], is best compatible with the idea that the site of action of Rho proteins in our system is close to the PLC-induced formation of Ins(1,4,5)P_3. In addition, and more importantly, the crucial effect of compactin on the tyrosine phosphorylation of PLCγ1 favours the idea that this enzyme is a direct or indirect target for active Rho proteins in the pathway of LTD_4-induced Ca^{2+} mobilization in epithelial cells. In regard to this and the observation by Chong and co-workers [36] it should be pointed out that a monomeric G-protein and a corresponding GTPase-activating protein (GAP) have also been implicated in the thrombin-induced regulation of PLCγ in human platelets [38].

Additional indirect support and a possible mechanism for the participation of Rho proteins in the regulation of PLC was provided by the cloning of a novel p122-RhoGAP, a protein that binds to and increases the activity of PLCδ [39]. The cited authors presumed that this p122-RhoGAP is involved in Rho signalling downstream of Rho activation [39]. However, as of yet there is no evidence that a direct interaction occurs between PLCδ and Rho proteins [39], nor could we find any support for such a direct association between Rho proteins and PLCγ1 in the present study. In analogy, it should be called to attention that studies on how Rho proteins regulate platelet phosphoinositide 3-kinase have been unable to demonstrate that Rho proteins are directly associated with the 3-kinase, which may imply that the action of Rho is indirectly mediated by some other molecule or too transient to be detected also in this system [40]. One possibility in the present investigation could be that the active Rho protein triggers the activation of the tyrosine kinase responsible for the phosphorylation of PLCγ1. This is suggested by the finding that compactin impaired the LTD_4-induced tyrosine phosphorylation of PLCγ1 and by the recent discovery that activation of the seven-transmembrane receptors for bombesin and endothelin respectively trigger signal transduction pathways in which Rho proteins act upstream of protein tyrosine phosphorylations [41,42]. Whether a p122-RhoGAP and/or a tyrosine kinase explains our failure to detect a direct interaction between Rho proteins and PLCγ1, or whether such an interaction was just too subtle and transient to be detected with the immunoprecipitation technique used, remains to be investigated.

In summary, in its ligand-bound form, the LTD_4 receptor of epithelial cells regulates the initial mobilization of intracellular Ca^{2+} via a direct or indirect Rho-induced activation of PLCγ1. This concept is compatible with the previous observations [12] that a pertussis toxin-sensitive G-protein is involved in the LTD_4-induced cellular influx of Ca^{2+} whereas a pertussis toxin-insensitive G-protein is involved in the intracellular mobilization of Ca^{2+}.

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REFERENCES

1 Samuelsson, B., Dahlén, S.-E., Lindgren, J.Å., Rouzer, C. A. and Serhan, C. N. (1987) Science **237**, 1171–1175
2 Chen, X.-S., Sheller, J. R., Johnson, E. N. and Funk, C. D. (1994) Nature (London) **372**, 179–182
3 Ford-Hutchinson, A. W. (1985) Fed. Proc. **44,** 25–29
4 Mong, S., Wu, H.-L., Hogaboom, G. K., Clark, M. A., Stadel, J. M. and Crooke. S. T. (1985) Eur. J. Pharmacol. **106**, 241–253
5 Badr, K. F., Mong, S., Hoover, R. L., Schwartzberg, M., Ebert, J., Jacobson, H. R. and Harris, R. C. (1989) Am. J. Physiol. **257**, F280–F287
6 Watanabe, T., Shimizu, T., Miki, I., Sakanaka, C., Honda, Z.-I., Seyama, Y., Teramoto, T., Matsushima T., Ui, M. and Kurokawa, K. (1990) J. Biol. Chem. **265**, 21237–21241
7 Hirasawa, N., Hoshi, T., Kudoh, M., Mue, S., Tsurufuji, S., Watanabe, M. and Ohuchi, K. (1992) Int. Arch. Allergy Immunol. **99**, 107–111
8 Berridge, M. J. (1993) Nature (London) **361**, 315–325
9 Blank, J. L., Brattain, K. A. and Exton, J. H. (1992) J. Biol. Chem. **267**, 23069–23075
10 Metters, K. M., Frey, E. A. and Ford-Hutchinson, A. W. (1991) Eur. J. Pharmacol. **194**, 51–61
11 Crooke, S. T., Mattern, M., Sarau, H. M., Winkler, J. D., Balcarek, J., Wong, A. and Bennett, C. F. (1989) Trends Pharmacol. **3**, 103–107
12 Sjölander, A., Grönroos, E., Hammarström, S. and Andersson, T. (1990) J. Biol. Chem. **265**, 20976–20981
13 Grönroos, D., Schippert, Å., Engström, M. and Sjölander, A. (1995) Cell Calcium **17**, 177–186
14 Marrero, M. B., Paxton, W. G., Duff, J. L., Berk, B. C. and Bernstein, K. E. (1994) J. Biol. Chem. **269**, 10935–10939
15 Jones, T. L. Z., Simonds, W. F., Merendino, J. J., Jr., Brann, M. R. and Spiegel, A. M. (1990) Proc. Natl. Acad. Sci. U.S.A. **87,** 568–572
16 Mumby, S. M., Casey, P. J., Gilman, A. G., Gutowski, S. and Sternweis, P. C. (1990) Proc. Natl. Acad. Sci. U.S.A. **87,** 5873–5877
17 Simonds, W. F., Butrynski, J. E., Gautam, N., Unson, C. G. and Spiegel, A. M. (1991) J. Biol. Chem. **266**, 5363–5366
18 Maltese, W. A. (1990) FASEB J. **4**, 3319–3328
19 Hall, A. (1994) Annu. Rev. Cell. Biol. **10**, 31–54
20 Cox, A. D. and Der, C. J. (1992) Curr. Opin. Cell. Biol. **4**, 1008–1016
21 Alberts, A. W., Chen, J., Kuron, G., Hunt, V., Huff, J., Hoffman, C., Rothrock, J., Lopez, M., Joshua, H., Harris, E., Patchett, A., Monaghan, R., Currie, S., Stapley, E., Albers-Schonberg, G., Hensens, O., Hirshfield, J., Hoogsteen, K., Liesch, J. and Springer, J. (1980) Proc. Natl. Acad. Sci. U.S.A. **77**, 3957–3961
22 Gibbs, J. B. (1991) Cell **65**, 1–4
23 Henle, G. and Deinhardt, F. (1957) J. Immunol. **25**, 54–59
24 Särndahl, E., Lindroth, M., Bengtsson, T., Fällman, M., Gustavsson, J., Stendahl, O., and Andersson, T. (1989) J. Cell Biol. **109**, 2791–2799
25 Grynkiewicz, G., Poenie, M. and Tsien, R. Y. (1985) J. Biol. Chem. **260**, 3440–3450
26 Mumby, S. M., Heukeroth, R. O., Gordon, J. I. and Gilman, A. G. (1990) Proc. Natl. Acad. Sci. U.S.A. **87,** 728–732
27 Laemmli, U. K. (1970) Nature (London) **227**, 680–685
28 Putney, J. W., Jr. (1990) Cell Calcium **11**, 611–624
29 Aktories, K. and Hall, A. (1989) Trends Pharmacol. Sci. **10**, 415–418
30 Deanin, G. G., Cutts, J. L., Pfeiffer, J. R. and Oliver, J. M. (1991) J. Immunol. **146**, 3528–3535
31 Li, G., Regazzi, R., Roche, E. and Wollheim, C. B. (1993) Biochem. J. **289**, 379–385
32 Jalink, K., van Corven, E. J., Hengeveld, T., Morii, N., Narumiya, S. and Moolenaar, W. H. (1994) J. Cell Biol. **126**, 801–810
33 Haag, H., Grünberg, B., Weber, C., Vaut, F., Aepfelbacher, M. and Seiss, W. (1994) Cell. Signal. **6**, 735–742
34 Zhang, J., King, W. G., Dillon, S., Hall, A., Feig, L. and Rittenhouse, S. E. (1993) J. Biol. Chem. **268**, 22251–22254
35 Malcolm, K. C., Ross, A. H., Qui, R.-G., Symons, M. and Exton, J. H. (1994) J. Biol. Chem. **269**, 25951–25954
36 Chong, L. D., Traynor-Kaplan, A., Bokoch, G. M. and Schwartz, M. A. (1994) Cell **79**, 507–513
37 Siddiqi, A. R., Smith, J. L., Ross, A. H., Qui, R.-G., Symons, M. and Exton, J. H. (1995) J. Biol. Chem. **270**, 8466–8473
38 Torti, M. and Lapetina, E. G. (1992) Proc. Natl. Acad. Sci. U.S.A. **89,** 7796–7800
39 Homma Y. and Emori, Y. (1995) EMBO J. **14**, 286–291
40 Zhang, J., Zhang, J., Benovic, J. L., Sugai, M., Wetzker, R., Gout, I. and Rittenhouse, S. E. (1995) J. Biol. Chem. **270**, 6589–6594
41 Rankin, S., Morii, N., Narumiya, S. and Rozengurt, E. (1994) FEBS Lett. **354**, 315–319
42 Ridley, A. J. and Hall, A. (1994) EMBO J. **13**, 2600–2610

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