Leukotriene D₄ induces association of active RhoA with phospholipase C- γ 1 in intestinal epithelial cells

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It has been previously suggested that leukotriene-induced Ca²⁺ signalling is mediated through a Rho-dependent process, but neither direct activation of Rho nor a mechanism underlying such signalling has been reported. Accordingly, we used the Rhotekin binding assay to assess RhoA activation in intestinal epithelial cells and observed that RhoA was activated by leukotriene D₄ (LTD₄). We also found that, within 15 s, activation of RhoA by LTD₄ led to an increased association of RhoA with G-protein $\beta\gamma$ (G $\beta\gamma$) and phospholipase C- γ 1 (PLC- γ 1) in the plasma membrane, as evidenced by the results of co-immunoprecipitation, glutathione S-transferase (GST) pulldown assays, and confocal microscopy. Amounts of RhoA increased in both G β and PLC- γ 1 immunoprecipitates within 15 s of LTD₄ treatment. An interaction between RhoA, G $\beta\gamma$ and PLC- γ 1 is supported by our finding that a GST fusion protein of constitutively active RhoA (GST-RhoAV14) precipitated G $\beta\gamma$ and

PLC- γ 1 from cell lysates in an agonist-dependent manner. Such an association is also substantiated by our confocal immunofluorescence results, which revealed that LTD₄ induction increased co-localization of constitutively active RhoA and PLC- γ 1 to the plasma membrane of cells transfected with enhanced green fluorescent protein L63RhoA. Furthermore, microinjection of neutralizing RhoA antibodies, but not control antibodies, significantly reduced LTD₄-induced Ca²⁺ mobilization. Our results are the first to demonstrate a LTD₄-induced activation of RhoA and more importantly its association with PLC- γ 1, which are essential for the PLC- γ 1-mediated calcium mobilization.

Key words: Ca²⁺ mobilization, G-protein $\beta\gamma$ subunits, LTD₄, PLC- γ 1, RhoA.

INTRODUCTION

Members of the Rho family of GTPases have been implicated as pivotal regulators of several signalling networks that are activated by a wide variety of receptors. The GTPases affect many aspects of cell behaviour, including actin cytoskeleton dynamics, transcriptional regulation, cell cycle progression, and membrane trafficking [1–3]. Rho is activated upon association with guanine nucleotide exchange factors (GEFs), which stimulate GTP binding to Rho in exchange for GDP [4]. Activation of members of the Rho family results in their translocation from the cytosol to the plasma membrane and subsequent interaction with a target and/or regulatory protein [5,6], few of which have been identified.

The GTPase Rho has also been implicated in agonist-induced Ca²⁺ signalling. Initially, Rho was shown to play a role in Ca²⁺ mobilization induced by platelet-derived growth factor and thrombin in C3H 10T1/2 cells and Swiss 3T3 cells, by regulating the activity of phosphatidylinositol 4-phosphate 5-kinase [7,8]. We found that leukotriene D₄ (LTD₄)-induced phospholipase C- γ 1 (PLC- γ 1) activation, inositol 1,4,5-trisphosphate [Ins(1,4,5)P₃] production, and Ca²⁺ mobilization were blocked by compactin, an inhibitor of isoprenylation, and the LTD₄-effected calcium signal was abolished by electroporated C3 exoenzyme in intestinal epithelial cells [9]. These results indirectly suggested that Rho participates in the regulation of this Ca²⁺ signal at the level of PLC- γ 1, but the antibodies we used in that study did not provide any support for a direct association between Rho and PLC- γ 1. In agreement with that, Rho activity has been shown to

be essential for Fc γ receptor-mediated protein tyrosine phosphorylation and calcium signalling in macrophages [10], whereas phosphatidic acid-induced Ca²⁺ mobilization has been reported to occur via a Rho-dependent but Ins(1,4,5)P₃-independent mechanism in rat-2 fibroblasts [11].

Leukotrienes (LTs), which are metabolites of arachidonic acid, are believed to mediate various pathological processes [12], and it has been shown that some of the effects of LTs depend on Rho [13,14]. For example, inhibitor experiments have suggested that LTs mediate growth-factor-induced Rho-dependent stress fibre formation [14]. Also, it was recently observed that the leukotriene designated LTD₄, which acts via the G-protein-coupled CysLT₁ receptor [15] induces C3 exoenzyme-dependent actin reorganization in human bronchial smooth muscle cells [13]. Furthermore, LTD₄ induces a calcium signal in intestinal epithelial cells that is regulated by two different G-proteins [16,17], one of which is responsible for LTD₄-induced Ca²⁺ mobilization and is insensitive to pertussis toxin, PTX [16]. We have also demonstrated that LTD₄ causes tyrosine phosphorylation and translocation of PLC- γ 1 to the membrane, indicating that it is activated [9]. As mentioned above, available data indirectly support a role for Rho in the regulation of LTD₄-induced Ca²⁺ mobilization, presumably at the level of PLC- γ 1. More recently, we further elucidated this signal by showing that LTD₄ triggers an increased association between PLC- γ 1 and G-protein $\beta\gamma$ (G $\beta\gamma$) subunits in intestinal epithelial cells [18]. In addition, we detected the widely expressed Src kinase c-Src in the G $\beta\gamma$ -PLC- γ 1 complex, suggesting that G $\beta\gamma$ subunits

Abbreviations used: EGFP, enhanced green fluorescent protein; G $\beta\gamma$, G-protein $\beta\gamma$; GEF, guanine nucleotide exchange factor; GST, glutathione S-transferase; Ins(1,4,5)P₃, inositol 1,4,5-trisphosphate; LT, leukotriene; LTD₄, leukotriene D₄; PLC- γ 1, phospholipase C- γ 1; RBD, Rho-binding domain of Rhotekin.

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act as a scaffold for molecules such as PLC- γ 1 and c-Src, which are involved in LTD₄-induced Ca²⁺ signalling in intestinal epithelial cells. In the cited study, we also observed that the Src kinase family inhibitor PP1 abolished LTD₄-induced Ca²⁺ mobilization, as well as LTD₄-induced tyrosine phosphorylation and association of PLC- γ 1 with G β γ .

G β γ subunits of heterotrimeric G-proteins appear to be involved in regulation of Rho family members, because studies have shown that dominant negative mutants of Rho proteins inhibit both G β γ -dependent activation of stress fibre formation in HeLa cells [19] and muscarinic activation of c-Jun N-terminal kinase, JNK [20]. However, little is known about G β γ -dependent signalling pathways to Rho proteins. It has been reported that G β γ subunits interact directly with Rho family proteins in a cell-free system [21]. Using a yeast two-hybrid system, Alberts et al. [22] demonstrated that the G β subunit can interact with RhoA in a GTP-dependent manner via ROCK-I/Kinectin homology sequences, suggesting a role for these subunits in recruiting Rho to the plasma membrane. It was recently shown that the Dbl family of RhoGEFs bind to G β γ subunits [23] through a pleckstrin homology (PH) domain-independent interaction, which further supports the concept of regulation/recruitment of Rho by G β γ subunits.

Considering the findings mentioned above, our present objective was to elucidate if and how Rho activation participates in the regulation of LTD₄-induced calcium mobilization in intestinal epithelial cells.

EXPERIMENTAL

Reagents

The cDNAs for the Rho-binding domain of Rhotekin (RBD) and plasmids containing GST-RhoAV14, L63RhoA, and N19RhoA were kindly provided by Dr M. A. Schwartz (Scripps Research Institute, La Jolla, CA, U.S.A.) and Dr A. Hall (Imperial Research Fund, London, U.K.) respectively. The anti-G β and anti-PLC- γ 1 antibodies were from Upstate Biotechnology (Lake Placid, NY, U.S.A.); anti-RhoA antibodies and ECL[®] reagents were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). LTD₄ was purchased from Cayman Chemical Company (Ann Arbor, MI, U.S.A.). Alexa 546 goat anti-mouse IgG and fura-2/AM were from Molecular Probes (Eugene, OR, U.S.A.). All other chemicals were of analytical grade and obtained from Sigma (St. Louis, MO, U.S.A.).

Cell culture

Human intestinal epithelial cells of the Int 407 line [24], which exhibit typical epithelial morphology and growth, were cultured for 5 days as a monolayer to approx. 80% confluence in Eagle's basal medium supplemented with 15% newborn-calf serum, 55 μ g/ml streptomycin, and 55 units/ml penicillin. The cells were incubated at 37 °C in a humidified atmosphere of 5% CO₂/95% air and were regularly tested to ensure the absence of mycoplasma.

Cell fractionation

The cells were washed and allowed to rest for 30 min in calcium medium (136 mM NaCl, 4.7 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 5.0 mM NaHCO₃, 20 mM Hepes, 1.0 mM CaCl₂ and 5.5 mM glucose, pH 7.4) as described in [16]. To prepare cytosolic and membrane fractions, the cells were scraped from the flasks into ice-cold buffer A [9], containing 20 mM Hepes, pH 8, 2 mM MgCl₂, 1 mM EDTA, 2 mM Na₃VO₄, 4 μ g/ml leupeptin, and 30 μ g/ml PMSF. The cells were homogenized, and heavier debris

was sedimented by centrifugation at 200 *g* for 10 min at 4 °C. The supernatants were then centrifuged at 1000 *g* for 5 min and subsequently twice at 200 000 *g* for 30 min. The cells were washed with calcium medium in between each centrifugation. The pellets were solubilized and used as membrane fractions.

Immunoprecipitation

The solubilized membrane fractions [18] were incubated for 1 h at 4 °C with anti-G β or anti-PLC- γ 1 antibodies and then for an additional hour with protein A/G-Sepharose beads. Immunoprecipitates were recovered by centrifugation at 10 000 *g* for 1 min and then washed (four times) with buffer A before further processing.

Gel electrophoresis

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

Immunoblotting

The separated proteins were electrophoretically transferred to a PVDF membrane. The membrane was blocked overnight at 4 °C with BSA (3% w/v) and subsequently incubated for 2 h at 20 °C with specific antibodies to PLC- γ 1 (1:500 dilution), RhoA (1:500 dilution), or G β (1:1000 dilution). Thereafter the PVDF membrane was washed extensively, incubated for 1 h at 20 °C with peroxidase-linked antibodies (dilution 1:10 000; Dako A/S, Glostrup, Denmark), and then washed and incubated with SuperSignal (Pierce, Rockford, IL, U.S.A.). The signal was revealed with a Fluor-S quantitative system (Bio-Rad Laboratories, Hercules, CA, U.S.A.).

Rho assay

LTD₄-stimulated and unstimulated cells were lysed in a lysis buffer [50 mM Tris/HCl, pH 7.5, containing 1 mM EDTA, 500 mM NaCl, 10 mM MgCl₂, 1% (v/v) Triton-X 100, and protease inhibitors (4 μ g/ml leupeptin and 30 μ g/ml PMSF)]. Cell lysates were clarified by centrifuging at 14 000 *g* for 10 min at 4 °C, and equal volumes of lysates (1 mg/ml) were incubated with glutathione-agarose beads (Sigma) coupled with bacterially expressed GST-RBD fusion protein [25] at 4 °C for 45 min. The beads were washed three times with lysis buffer (supplemented with 150 mM NaCl). Bound Rho proteins were detected using an antibody against RhoA. The amount of RBD-bound Rho was normalized to the Rho content in cell lysates.

GST fusion proteins and binding assays

The cDNA clone encoding the GST fusion proteins of RhoAV14 in the pGEX vector (Glutathione S-transferase Gene fusion vector; Amersham Biosciences, Uppsala, Sweden) was transformed into *Escherichia coli* and cultured at 30 °C. Expression of GST fusion proteins was induced by 1 mM isopropyl β -D-thiogalactopyranoside. The cells were subsequently collected by centrifugation at 3500 *g* for 15 min, sonicated in PBS, and clarified by centrifugation for 15 min at 10 000 *g*. The clarified lysate was incubated with glutathione-agarose beads for 1 h at 4 °C and then washed (three times) with ice-cold PBS. Thereafter, the bound protein was eluted with 10 mM reduced glutathione in

50 mM Tris/HCl, pH 8.0, and concentrated on a Centricon-10 column (Amicon, Geneva, Switzerland). To analyse proteins binding to GST fusion protein, lysates of LTD₄-stimulated and unstimulated cells were prepared in 1.0 ml of lysis buffer [18]. GST fusion proteins or agarose beads with prebound GST were incubated with lysates (1 mg/ml of protein) for 2 h at 4 °C. Thereafter the beads were washed (three times) with ice-cold lysis buffer, and solubilized proteins were subjected to Western blotting.

Construction and transfection of enhanced green fluorescent protein (EGFP)-RhoA in Int 407 cells

cDNAs coding for L63RhoA and N19RhoA were originally obtained from Dr Alan Hall (MRC Laboratory, London, U.K.) and cloned into the pEGFP-N1 vector (Clontech Laboratories, Erembodegem, Belgium). The cells were subjected to transient transfection using 3.5 μ l of Lipofectin (Gibco BRL, Basel, Switzerland) and 1.8 μ g of plasmid DNA/ml in serum-free medium, essentially according to the protocol provided by the supplier.

Confocal microscopy

The cells were seeded on to glass coverslips, grown for 5 days (serum-starved for the last 16 h), and then exposed to 40 nM LTD₄ in a tissue culture incubator at 37 °C. The LTD₄ treatment was terminated by fixing in PBS containing 3.7% (v/v) paraformaldehyde for 10 min at 20 °C. The cells were subsequently permeabilized in PBS containing 0.5% (v/v) Triton X-100 for 5 min, after which the coverslips were washed twice in PBS and incubated at 20 °C in PBS containing 3% (v/v) BSA for 15 min. The cells were then stained for 1 h with specific antibodies against PLC γ -1 (5 μ g/ml). Thereafter, the coverslips were washed six times in PBS and incubated with a 1:200 dilution (in blocking buffer) of Alexa 546 goat anti-mouse IgG secondary antibody. As a negative control, we used normal mouse IgG. Finally the coverslips were washed six times in PBS and mounted using a fluorescent mounting medium (Dako A/S). Images were obtained using a Bio-Rad Radiance 2000 confocal laser scanning system with a Nikon microscope (model TE300) equipped with a 60 $\times$ 1.4 Plan-APOCHROMAT oil immersion objective.

Calcium imaging

Cells were incubated with 4 μ M fura-2/AM in culture medium for 30 min at 37 °C [16]. Thereafter, the cells were placed in a chamber containing calcium-free medium (supplemented with 1 mM EGTA) as described previously [16]. The Ca²⁺-imaging system consisted of a Nikon Diaphot microscope connected to a Photon Technology International (PTI) Image System [18]. Images were acquired by exciting the cells at 340 and 380 nm, while recording the emission at 510 nm, before and after stimulation with LTD₄. The fluorescence intensity ratios (340/380 nm) of the images were subsequently analysed after background subtraction, using PTI Image Master software.

Microinjection

The cells were microinjected with C3 exoenzyme (0.06 mg/ml) or anti-RhoA antibodies (0.2 mg/ml) or with isotypic control antibodies, using an Eppendorf Micromanipulator 5171/Trans-jector 5246 (injection pressure 150–200 hPa; injection time 0.7 s) as described previously [18]. Prior to injection, the antibody solutions were clarified by centrifuging at 10000 g for 20 min.

RESULTS

Activation of Rho by LTD₄

As a first step in elucidating the role of RhoA in LTD₄ signalling [9], we used GST-RBD, which binds specifically to active Rho (GTP-Rho [25]), to determine whether LTD₄ can activate RhoA. The basal level of RhoA activity in the Int 407 cells was rapidly (within 15 s) increased by LTD₄, and the activity returned to basal within 300 s (Figure 1A). Densitometric analysis revealed an approx. 3.5-fold increase in RhoA bound by GST-RBD (Figure 1B). As an assay control, we treated the cells with the specific Rho activator cytotoxic necrotizing factor-1 [26] from the *E. coli* strain BM2-1 (a gift from Professor G. Donelli; Figure 1A). We also confirmed the specificity of binding of GST-RBD to active Rho by using constitutively active RhoA (RhoA^{V14}) and wild-type inactive RhoA (results not shown).

LTD₄ caused RhoA to associate with G β γ and PLC- γ 1

It is known that activation of Rho leads to translocation of this protein from the cytosol to the plasma membrane [5]. To determine whether active RhoA can interact with G β γ and PLC- γ 1, we performed separate immunoprecipitations of the G β subunits and PLC- γ 1 in the solubilized membrane fractions and then immunoblotted for RhoA. We found a low basal association between G β subunits and RhoA, which was increased within 15 s of exposure to LTD₄ (Figure 2A). Densitometric analyses revealed that the association was increased by 50% and that it decreased after 300 s (Figure 2A). We also found RhoA in the PLC- γ 1 immunoprecipitates, and the level of this protein was increased 2.5-fold by treatment with LTD₄ (Figure 2B). To

Figure 1 Activation of RhoA by LTD₄ in intestinal epithelial cells

(A) Cells were stimulated with 40 nM LTD₄ for the indicated periods of time and then lysed, and lysates were assayed for Rhotekin-binding activity as described in the Experimental section. Cell lysates were assayed for RhoA activity by RBD binding. For these experiments, the total RhoA bound to the RBD (top panel in A) was normalized to the RhoA content of cell lysates (bottom panel in A). CNF1, cytotoxic necrotizing factor-1. The blots shown are representative of three separate experiments. (B) Statistical significance of densitometric data on the RBD immunoprecipitation experiments was analysed by Student's *t* test, **P* < 0.05.

Figure 2 Interactions between $G\beta\gamma$ and PLC- γ 1 with RhoA in a membrane fraction

Cells were stimulated with 40 nM LTD₄ for the indicated periods of time and then lysed and fractionated. (A) Anti- $G\beta$ immunoprecipitates (IP: $G\beta$), or (B) anti-PLC- γ 1 immunoprecipitates (IP: PLC- γ 1) were separated by SDS/PAGE and immunoblotted with anti-RhoA or, as a control, with anti- $G\beta$ or PLC- γ 1 antibodies. Immunoprecipitation with isotype-matched non-immune control IgG antibodies was done in all experiments to exclude the possibility of unspecific associations (N). The blots shown are representative of at least four separate experiments. Statistical significance of densitometric data on the immunoprecipitation experiments was analysed by Student's *t* test, **P* < 0.05.

confirm these associations, we performed binding assays using a constitutively active GST-RhoV14 fusion protein, because $G\beta$ in a cell-free system has been shown to interact solely with GTP-RhoA [21]. $G\beta$ subunits were precipitated from the cell lysates by GST-RhoAV14 as illustrated in Figure 3(A), but not by GST alone (results not shown). We detected an interaction between $G\beta$ and GST-RhoAV14 in unstimulated lysates, and this interaction was significantly increased by exposure to LTD₄. We also found that GST-RhoAV14 protein caused increased precipitation of PLC- γ 1 from LTD₄-stimulated cell lysates (Figure 3B). This RhoAV14-mediated precipitation of PLC- γ 1 was completely inhibited by the Src family tyrosine-kinase inhibitor PP1. Taken together with our earlier finding that PP1 also blocks the association of $G\beta$ subunits and PLC- γ 1 [18], these data indicate an intimate relationship between these three proteins in the membrane fraction.

To gain additional evidence of an association between these proteins, we examined further the influence of LTD₄ on the distribution of RhoA and PLC- γ 1 in cells transfected with either the constitutively active RhoA (EGFP-L63RhoA) or the dominant negative RhoA (EGFP-N19RhoA). In unstimulated cells, PLC- γ 1 and EGFP-N19RhoA were distributed fairly homogeneously throughout the cytoplasm, whereas EGFP-L63RhoA was distinctly localized to the plasma membrane around the cell margin. We observed a low amount of PLC- γ 1 associating with active RhoA in unstimulated cells (Figure 4A). Treatment with LTD₄ for 1 min resulted in translocation of PLC- γ 1 to the plasma membrane and colocalization with EGFP-L63RhoA at the periphery of the plasma membrane around the cell margin (Figure 4A). In EGFP-N19RhoA-transfected cells, LTD₄ stimu-

Figure 3 Interactions between PLC- γ 1 and RhoA in GST pull-down experiments

The cells were stimulated with 40 nM LTD₄ for the indicated periods of time and then lysed. The lysates were clarified, and aliquots were incubated with GST-RhoAV14 or GST alone; bound proteins were separated by SDS/PAGE and immunoblotted with (A) anti- $G\beta$ ($G\beta$) or (B) anti-PLC- γ 1 (PLC- γ 1) or were incubated with anti-GST (GST-RhoA) antibodies as a control. In (B), the cells were pretreated with the Src family kinase inhibitor PP1 (10 μ M) for 30 min before stimulation with 40 nM LTD₄ for 15 s. The blots shown are representative of at least three separate experiments.

lation also resulted in translocation of PLC- γ 1 to the cell membrane, but there was no colocalization with EGFP-N19RhoA (Figure 4B). These results demonstrate that LTD₄ induces translocation and association of active RhoA with PLC- γ 1 at the cell membrane.

Effect of RhoA inhibition on LTD₄-induced Ca²⁺ mobilization

To evaluate the physiological significance of RhoA activation in the LTD₄-induced Ca²⁺ signal, we inhibited RhoA by microinjecting specific antibodies against RhoA before assessing the effects on LTD₄-induced Ca²⁺ mobilization in single cells. We found that the mobilization was significantly inhibited by specific antibodies that neutralized RhoA (Figure 5), but it was not changed by microinjection of constitutively active RhoA, GST-RhoAV14, or isotype-matched control antibodies (results not shown). As an additional control we also microinjected C3 exoenzyme. Together, these findings suggest that activation of RhoA is essential for LTD₄-induced Ca²⁺ mobilization.

DISCUSSION

Our study is the first to reveal an agonist-induced association of active RhoA with $G\beta\gamma$ and PLC- γ 1 in intact cells. Our experiments also led to the novel observations that, in intestinal epithelial cells, LTD₄ causes an activation and association of RhoA with PLC- γ 1 that are essential for the downstream PLC- γ 1-mediated calcium signal.

Figure 4 Effects of LTD₄ on colocalization of EGFP-RhoA and PLC- γ 1

Cells were transfected with constitutively active EGFP-RhoA (L63RhoA) or the dominant negative of EGFP-RhoA (N19RhoA) and then stimulated with LTD₄, fixed, permeabilized, and stained for PLC- γ 1 as described in the Experimental section. The illustrated results are representative of five separate experiments. **(A)** Cellular distribution of EGFP-L63RhoA and PLC- γ 1 and an overlay image (Merge; colocalization shown in white) are shown for cells not stimulated with LTD₄ (upper panels) and for cells treated with 40 nM LTD₄ (lower panels). **(B)** As in **A**, but the cells were transfected with EGFP-N19RhoA in the absence (upper panels) or presence (lower panels) of 40 nM LTD₄.

Figure 5 Single-cell calcium traces obtained from representative images of fura-2-loaded cells stimulated after 15 s with LTD₄

(A) The traces represent non-microinjected cells (C), cells microinjected with an isotypic control antibody (C Ab), cells microinjected with C3-exoenzyme (C3) and cells microinjected with neutralizing anti-RhoA antibodies (RhoA Ab). (B) Bars indicate changes in the ratio 340/380 nm, ($\pm$ S.E.M.) after exposure to LTD₄, based on three separate experiments.

In G-protein-coupled receptor signalling systems, both α and $\beta\gamma$ subunits of G-proteins interact with specific targets and thereby initiate downstream signalling cascades. Concerning $G\alpha$ signalling and activation of Rho family members, Hart and coworkers [27] observed that activated $G\alpha_{13}$ bound tightly to p115-RhoGEF, whereas activated $G\alpha_{12}$ impaired this association. Furthermore, the same research group [28] found that recombinant p115-RhoGEF increased the GTPase activity of $G\alpha_{12}$ and $G\alpha_{13}$ in a cell-free system. Hence there is evidence of a significant link between certain active $G\alpha$ proteins and a regulatory element of the small GTP-binding protein Rho. In regard to $G\beta\gamma$ signalling, Nishida et al. [23] recently found that $G\beta\gamma$ subunits can associate with members of the Dbl family of RhoGEFs in transient co-expression and cell-free systems. However, in that investigation, the GEF activity of Dbl towards Rho was not affected by $G\beta\gamma$ binding, which implies that additional and/or alternative signals may be required for $G\beta\gamma$ signalling to Rho. These findings suggest that $G\beta\gamma$ subunits can regulate the function of Rho by recruiting RhoGEFs to the membrane. Harhammer and colleagues [21] studied a cell-free system and noted that $G\beta\gamma$ subunits interacted directly with Rho, and other investigators have found that $G\beta$ subunits also interact with

RhoA in a yeast two-hybrid system [22]. However, there are no reports indicating receptor-mediated interaction of $G\beta\gamma$ and Rho in intact cells.

In a previous study of epithelial cells [18], we demonstrated that LTD₄ triggers an association between $G\beta\gamma$, PLC- γ 1 and c-Src that is important for the downstream Ca²⁺ signal, which implies that $G\beta\gamma$ acts as a scaffold protein for these signalling molecules. In light of these findings, we investigated a possible agonist-mediated interaction of RhoA with $G\beta\gamma$ and PLC- γ 1, and found that exposing cells to LTD₄ caused increased association of RhoA with $G\beta\gamma$ and PLC- γ 1 in the membrane fraction. Moreover, a GST fusion protein of constitutively active RhoA (GST-RhoAV14) precipitated only a small amount of $G\beta$ and PLC- γ 1 from unstimulated cell lysates, but a significant amount from LTD₄-treated lysates. These results suggest that $G\beta\gamma$ subunits can participate in the translocation of active RhoA to the plasma membrane. $G\beta\gamma$ subunits have been shown to bind and, often, also to regulate different effector molecules through pH-domain-mediated interactions [29]. However, regarding possible attachment of $G\beta\gamma$ to RhoA, experiments in a two-hybrid system have shown that a ROCK-I/Kinectin homology region in $G\beta$ can bind directly to active RhoA [22]. These indications of a possible direct interaction between $G\beta\gamma$ and RhoA are compatible with our presented results indicating a role of $G\beta\gamma$ in the membrane targeting of active RhoA.

Our previous results, concerning the effects of the isoprenylation inhibitor compactin and C3 exoenzyme on LTD₄-induced PLC- γ 1 activation and Ca²⁺ mobilization, suggested a regulatory role for Rho at the level of PLC- γ 1 [9]. However, the antibodies that were available to immunoprecipitate these proteins at that time, could not reveal any physical association between RhoA and PLC- γ 1 in whole-cell lysates [9]. By comparison, in the present study we were able to demonstrate an association between RhoA and PLC- γ 1 by three different methods. First we found an increased RhoA content in PLC- γ 1 immunoprecipitates from membrane fractions of LTD₄-stimulated cells. Secondly, we found that LTD₄ induced colocalization of PLC- γ 1 with active EGFP-L63RhoA in the plasma membrane of intact cells, as revealed by confocal microscopy. Third, we found that GST pulldown experiments, with constitutively active RhoA, precipitated PLC- γ 1 from lysates of LTD₄-stimulated cells. The LTD₄-induced activation and subsequent association of RhoA with PLC- γ 1 is evidently important for the regulation of PLC- γ 1 activity and the downstream calcium mobilization. This was demonstrated since microinjection of C3 exoenzyme or neutralizing antibodies against RhoA impaired the LTD₄-induced calcium mobilization. The present observations are partly supported by a recent report showing that transfection of constitutively active Cdc42 led to increased antigen-induced Ins(1,4,5)P₃ production and calcium mobilization in RBL-2H3 mast cells [30], probably by its binding to PLC- γ 1, as indicated by GST-Cdc42 pulldown assays. Despite the fact that the PLC- δ isoform is not tyrosine phosphorylated, but is instead activated by some other mechanism, our results are also in part substantiated by data published by Hodson et al. [31], showing that RhoA can be co-immunoprecipitated with PLC- δ from bovine aorta, where it acts as a negative modulator of PLC- δ activity. Clearly, the present results do not indicate whether the interaction between PLC- γ 1 with RhoA is direct or indirectly mediated by $G\beta\gamma$ subunits. However, our data show that only the active forms of RhoA (GTP-bound) and PLC- γ 1 (tyrosine phosphorylated) can be included in the LTD₄-induced protein signalling complex at the plasma membrane.

The present findings, that RhoA is activated by LTD₄ and becomes associated with PLC- γ 1, would suggest that the $G\beta\gamma$

subunit targets RhoA and PLC- γ 1 to the plasma membrane by acting as a scaffold protein and thereby enabling their interaction. Such a scenario can explain, in part, how RhoA participates in the regulation of LTD₄-induced Ca²⁺ mobilization at the level of PLC- γ 1.

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