Leukotriene D_4 induces stress-fibre formation in intestinal epithelial cells via activation of RhoA and PKCδ

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Summary

The intestinal epithelial barrier, which is regulated by the actin cytoskeleton, exhibits permeability changes during inflammation. Here we show that activation of the $CysLT_1$ receptor by the inflammatory mediator leukotriene D_4 (LTD_4) causes a rapid increase in stress-fibre formation in intestinal epithelial cells. This effect was mimicked by cytotoxic necrotising factor-1 (CNF-1)-induced activation of RhoA, overexpression of constitutively active RhoA (L63-RhoA) and phorbol-ester-induced activation of protein kinase C (PKC). In accordance, inhibition of RhoA, by C3 exoenzyme or by dominant-negative RhoA (N19-RhoA), as well as GF109203X-induced inhibition of PKC, suppressed the LTD_4-induced stress-fibre formation. Introduction of the dominant-negative regulatory domain of PKCδ, but not the corresponding structures from PKCα, βII or ε, blocked the LTD_4-induced stress-fibre formation. Evaluating the relationship between PKCδ and RhoA in LTD_4-induced stress-fibre formation, we found that C3 exoenzyme inhibited the rapid LTD_4-elicited translocation of PKCδ to the plasma membrane. Furthermore, CNF-1-induced stress-fibre formation was blocked by GF109203X and by overexpression of the regulatory domain of PKC-δ, whereas PKC-induced stress-fibre production was not affected by N19-RhoA. We conclude that PKC-δ is located downstream of RhoA and that active RhoA and PKCδ are both necessary for LTD_4-induced stress-fibre formation.

Key words: LTD_4, CNF-1, Stress fibres, RhoA, PKC-δ

Introduction

Leukotrienes (LTs) are essential mediators of several pathological processes, such as asthma and other hypersensitive reactions, as well as inflammatory bowel disease (Samuelsson et al., 1987; Hammerbeck and Brown, 1996). LTs are extremely potent compounds that induce a large number of functional effects, including increased vascular permeability, contraction of smooth muscle and mucus secretion (Samuelsson et al., 1987). During an inflammatory reaction, the intestinal epithelial cells are influenced by LTD_4 and other inflammatory mediators (Samuelsson et al., 1987). Earlier studies have demonstrated that rearrangement of filamentous actin (F-actin) is induced by different LTs, including LTD_4 (Welles et al., 1985; Bautz et al., 2001; Massoumi and Sjölander, 1998), but the mechanisms of this are not known.

Activation of the LTD_4 receptor $CysLT_1$ triggers a cascade of intracellular signalling events, including activation of the serine/threonine protein kinase C (PKC) (Thodeti et al., 2001; Vegesna et al., 1988). PKC can be classified into three major subgroups: the classical PKCs (PKCα, βI, βII and γ), which are Ca^{2+}/diacylglycerol dependent; the novel PKCs (PKCδ, ε, τ and η). which are Ca^{2+} independent but require diacylglycerol for activation; and the atypical PKCs ζ and ι, which are neither Ca^{2+} nor diacylglycerol dependent (Ron and Kazanietz, 1999). PKC isoforms are expressed to varying degrees in all mammalian cells (Martiny-Baron et al., 1993; Mellor and Parker, 1998; Nishizuka, 1992) and have been linked to changes in the cytoskeleton in many different cell types (for a review, see Keenan and Kelleher, 1998). If a PKC isoform(s) is involved in the LTD_4-induced changes of the actin cytoskeleton in intestinal cells, it has to be one of the α, βII, δ or ε isoforms, since these are the ones activated by this LT (Thodeti et al., 2001).

RhoA belongs to the family of Rho GTPases, which are key regulators of the actin cytoskeleton in many types of cells (Hall, 1998) and function as molecular switches by alternating between a GDP-bound inactive form and a GTP-bound active form (Hall, 1998). There are at least 14 Rho GTPases, which share more than 50% sequence identity, and the most extensively studied members of this family are RhoA, Cdc42 and Rac1 (Hall, 1998). RhoA regulates the formation of stress fibres and focal adhesions, whereas Cdc42 and Rac1 are believed to stimulate the formation of filopodia and lamellipodia, respectively (Ridley and Hall, 1992). The C3 exoenzyme from *Clostridium botulinum* and cytotoxic necrotising factor-1 (CNF-1) from *Escherichia coli* are important tools for studying the regulation of the actin cytoskeleton by their ability to regulate the Rho protein. Treatment with C3 exoenzyme, which is an ADP-ribosyltransferase that catalyses mono-ADP ribosylation of RhoA at Asp41, results in inhibition of RhoA and selective disorganisation of actin stress fibres (Chardin et al., 1989); exposure to CNF-1 induces the opposite effects. CNF-1

exhibits catalytic deamidase activity specific to Rho Gln63. Modification of Gln63 to Asp63 in Rho blocks the intrinsic and GAP-stimulated hydrolysis of GTP, resulting in permanent activation of the GTP-binding protein and thereby provoking prominent bundling of actin stress fibres (Flatau et al., 1997; Schmidt et al., 1997).

In the present study, we show that LTD_4 causes stress-fibre production via activation of PKCδ and a subsequent downstream activation of RhoA. To our knowledge, this is the first study to show that RhoA is involved in rearrangement of the actin cytoskeleton through a downstream effect on PKCδ.

Materials and Methods

Materials

The antibodies and their sources were as follows: Alexa 488 phalloidin and Alexa 546 phalloidin Molecular Probes Inc. (Eugene, OR); anti-PKCα, Upstate Biotechnology (Lake Placid, NY); anti-PKCδ and anti-PKCε, Santa Cruz Biotechnology (Santa Cruz, CA); Mouse IgG (normal), peroxidase-linked goat anti-mouse and goat anti-rabbit IgG, DAKO A/S (Glostrup, Denmark); LTD_4 was obtained from Cayman Chemical Co. (Ann Arbour, MI); 12-O tetradecanoylphorbol 13 acetate (TPA), Gö6976, GF109203X and *Clostridium botulinum* C3 exoenzyme were from Calbiochem-Novabiochem Co. (San Diego, CA). All other chemicals were of analytical grade and were obtained from Sigma Chemical Co. (St Louis, MO).

Cell culture

The intestinal epithelial cell line Intestine 407 (Henle and Deinhardt, 1957) was cultured as a monolayer in 75 cm^2 flasks in Eagle's basal medium supplemented with 15% new-born calf serum, 55 μg/ml streptomycin and 55 IU/ml penicillin. Cell cultures were kept at 37°C in a humidified environment of 5% CO_2 and 95% air. The cells, which exhibit typical epithelial morphology and growth, were regularly tested to ensure the absence of mycoplasma contamination.

Cell fractionation, gel electrophoresis and immunoblotting

The cells were serum-starved for 12 hours and then stimulated with 40 nM LTD_4 in the absence or presence of a mixture of 5 μg/ml lipofectamine and 5 μg/ml C3 exoenzyme for 10 hours as described elsewhere (Borbiev et al., 2000). The stimulation was terminated by adding ice-cold buffer A containing 20 mM NaHepes (pH 8), 2 mM $MgCl_2$ 1 mM EDTA, 2 mM Na_3VO_4, 4 μg/ml leupeptin and 30 μg/ml PMSF. Thereafter, the cells were scraped into the cold buffer, homogenised 10 times on ice in a glass tissue grinder (Dounce) and then centrifuged at 200 ***g*** for 10 minutes. The protein content of the supernatant was measured and compensated for, and the supernatant was subsequently centrifuged at 1000 ***g*** for 5 minutes. The supernatant of the 1000 ***g*** fraction was further centrifuged at 200,000 ***g*** for 30 minutes. The resulting membrane-rich pellet was suspended in 150 μl of buffer A, and boiled in SDS-sample buffer for 10 minutes and loaded onto 8% polyacrylamide gels. The separated proteins were transferred to PVDF membranes, which were blocked in 3% BSA/PBS and incubated for 1 hour at 25°C in a 1:500 dilution of PKCα, δ or ε antibodies in PBS with 3% BSA. The membranes were probed with a 1:5000 dilution of a secondary antibody coupled to horseradish peroxidase for 1 hour at 25°C and thereafter developed with an ECL kit (Amersham).

Confocal microscopy

The cells were seeded onto glass coverslips and grown for five days. These cells were serum starved for 12 hours and then stimulated with either 40 nM LTD_4 for 5 minutes or 100 nM TPA for 15 minutes in a tissue culture incubator at 37°C; this was done with or without pre-incubation with 2 μM GF109203X or 2 μM Gö6976 for 15 minutes, 300 ng/ml CNF-1 for 16 hours or a mixture of 5 μg/ml lipofectamine and 5 μg/ml C3 exoenzyme for 10 hours. The stimulations were terminated by fixing the cells for 10 minutes at room temperature in 3.7% paraformaldehyde/PBS solution, after which the cells were permeabilised in 0.5% Triton X-100/PBS solution for 5 minutes. The coverslips were subsequently washed twice in PBS and incubated at room temperature in 3% BSA/PBS solution for 15 minutes. The cells were then stained for 30 minutes with Alexa 488 phalloidin or Alexa 546 phalloidin (5 μg/ml). Thereafter, the coverslips were washed six times in PBS and mounted in a fluorescent mounting medium (DAKO A/S). Confocal images were recorded using a Bio-Rad Radiance 2000 confocal laser scanning system with a Nikon microscope (model TE300) equipped with a 60×1.4 Plan-APOCHROMAT oil immersion objective.

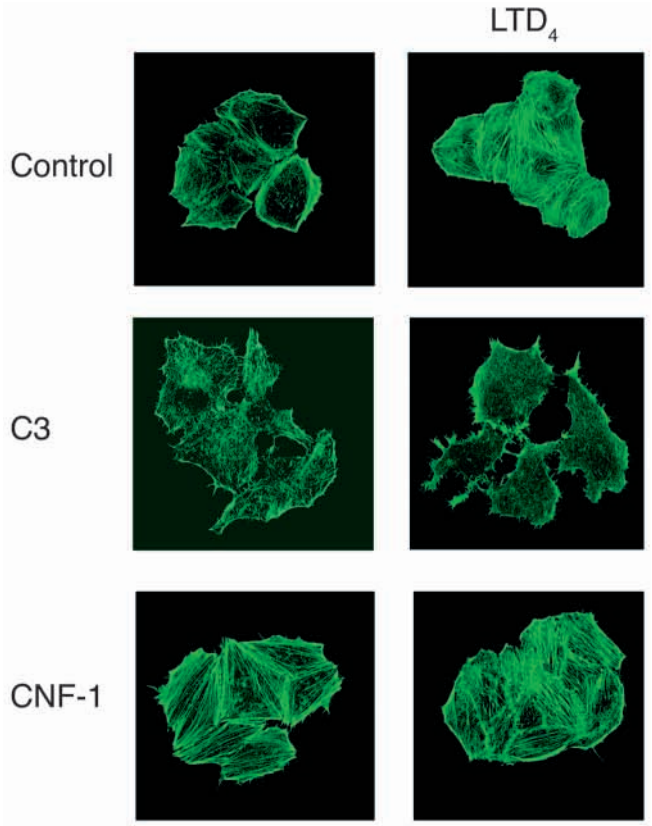

Fig. 1. Effects of LTD_4, CNF-1 and C3 exoenzyme on stress-fibre formation. Int 407 cells were incubated with or without LTD_4, C3 exozyme or CNF-1. The cells were subsequently fixed, permeablised, stained for F-actin with Alexa 488 phalloidin and examined by confocal microscopy. The upper two panels show unstimulated (Control) and LTD_4-treated cells. The two panels in the middle (C3) illustrate cells incubated with 5 μg/ml C3 exoenzyme and 5 μg/ml lipofectamine for 10 hours in the absence or presence of LTD_4. The lower two panels (CNF-1) show cells incubated with 300 ng/ml CNF-1 for 16 hours in the absence or presence of LTD_4. The results illustrated in this figure are representative of ten separate experiments.

Construction and transfection of EGFP-RhoA and EGFP-RD-PKC in Int 407 cells

L63-RhoA and N19-RhoA cDNAs were originally obtained from

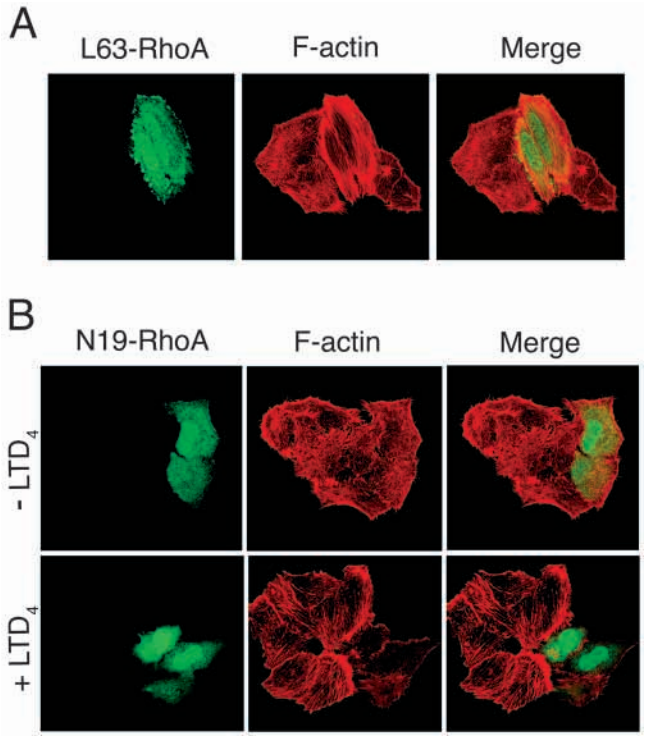

Fig. 2. Effects of L63-RhoA and N19-RhoA on stress-fibre formation. (A) Cells were transfected with EGFP-L63-RhoA and then fixed, permeablised, stained for F-actin with Alexa 546 phalloidin and examined by confocal microscopy. (B) Cells were transfected with EGFP-N19-RhoA in the absence or presence of 40 nM LTD_4 for 5 minutes and then processed as in A. Merged images are shown on the right. The results illustrated in this figure are representative of six separate experiments.

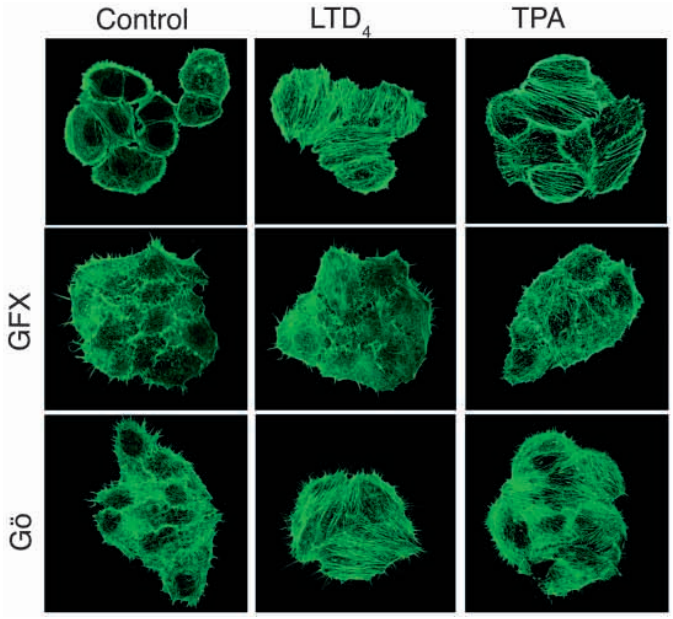

Fig. 3. Effects of LTD_4 and TPA on stress-fibre formation. Following stimulation with either 40 nM LTD_4 for 5 minutes or 100 nM TPA for 15 minutes, the cells were fixed, permeablised, stained for F-actin with Alexa 488 phalloidin and examined by confocal microscopy. The different panels show cells that were not stimulated (control) or stimulated with LTD_4 or TPA and also were or were not exposed to 2 μm GF109203X or 2 μm Gö6976 for 15 minutes. The results illustrated in this figure are representative of five separate experiments.

Alan Hall (MRC Laboratory, London) and cloned into the pEGFP-N1 vector (Clontech Laboratories, Inc.). Expression vectors encoded the regulatory domain of PKCα, βII, δ, ε or full-length PKCδ EGFP-fusion proteins (Zeidman et al., 1999). Transient transfections of the cells were performed using 5.0 μg/ml Lipofectin (GIBCO) and 1.8 μg of plasmid DNA/ml in serum-free medium, essentially according to the protocol provided by the supplier.

Results

LTD_4 induces stress-fibre formation via RhoA

We exposed the Int 407 cells (serum starved for 12 hours) to 40 nM LTD_4 for 5 minutes and then stained for F-actin (phalloidin) and examined the cells by confocal microscopy. Control cells that were not exposed to LTD_4 exhibited interior diffuse and punctuate phalloidin staining, as well as organised actin filament bundles around the plasma membrane (Fig. 1, top left). After 5 minutes of LTD_4 treatment, most cells showed increased aggregation of actin filament bundles and parallel arrays of stress fibres in the cells (Fig. 1, top right). We also observed that other human intestinal epithelial cell lines, such as FHs 74 and Caco-2, displayed the same pattern as the Int 407 cells upon exposure to 40 nM LTD_4 (data not shown). Activation of the small GTP-binding protein RhoA is involved in the formation of actin stress fibres (Ridley and Hall, 1992), thus we performed experiments to determine whether RhoA mediates stress-fibre formation in LTD_4-stimulated epithelial cells. We found that incubating the cells for 10 hours with the specific Rho GTPase inhibitor C3 exoenzyme (5 μg/ml), mixed with lipofectamin (5 μg/ml) to enable cell penetration (Borbiev et al., 2000), disrupted the LTD_4-induced stress-fibre formation (Fig. 1, middle panel). CNF-1 is a 110 kDa toxin produced by certain pathogenic strains of *E. coli*. This toxin induces the formation of actin stress fibres primarily by abolishing the ability of Rho to hydrolyse GTP into GDP (Flatau et al., 1997; Schmidt et al., 1997). After exposure to CNF-1 for 16 hours, the cells showed increased aggregation of stress fibres – stimulation with both LTD_4 and CNF-1 slightly increased the number of stress fibres compared with treatment with LTD_4 or CNF-1 alone (Fig. 1, lower panel). Fig. 2 shows Int 407 cells transfected with constitutively active EGFP-RhoA (L63-RhoA) or dominant-negative EGFP-RhoA (N19-RhoA). Cells transfected with L63-RhoA exhibited pronounced formation of stress fibres, whereas cells transfected with EGFP-N19-RhoA displayed no stress-fibre formation regardless of the absence (control) or the presence of 40 nM LTD_4 (Fig. 2B). Non-transfected cells displayed increased stress-fibre formation after stimulation with LTD_4 (Fig. 2B). In some cells a tendency to a nuclear accumulation of N19-RhoA was observed but that was independent of LTD_4 stimulation and most probably related to the level of expression of the mutated protein in transfected cells. These results suggest that activation of RhoA is necessary for the generation of stress fibres induced by LTD_4 in these cells.

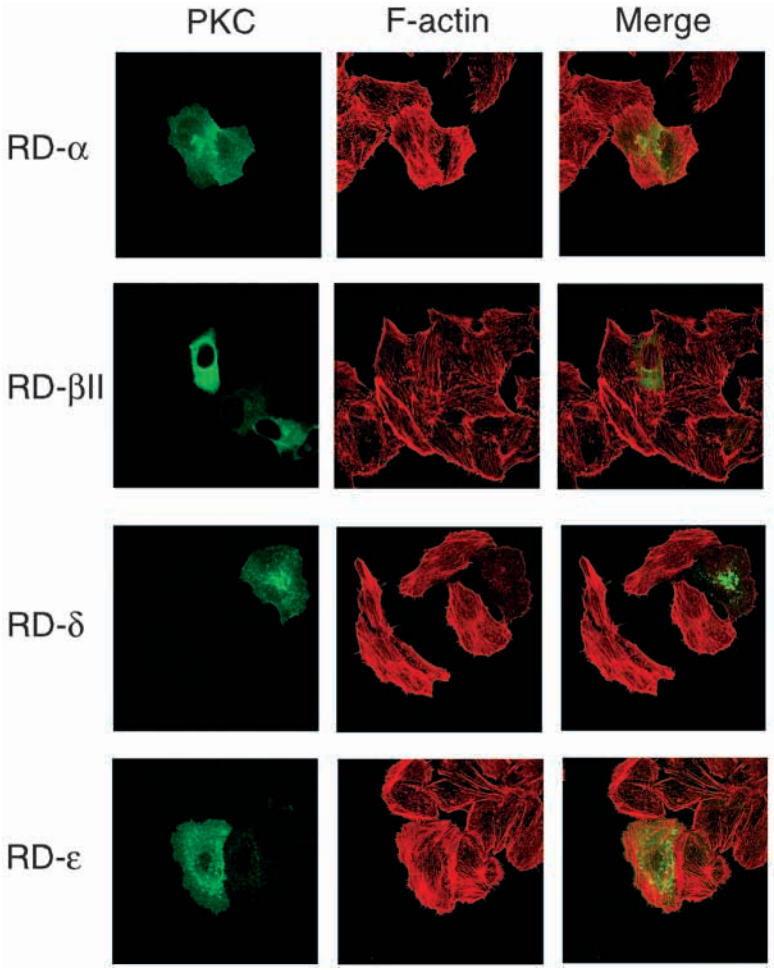

Fig. 4. Effects of different PKC regulatory domains (RD) on stress-fibre formation. Cells were first transfected with vectors encoding EGFP-RD-PKCα, –RD-PKCβII, –RD-PKCδ, or –RD-PKCε and then stimulated with 40 nM LTD_4 for 5 minutes. Following stimulation, the cells were fixed, permeablised, stained for F-actin with Alexa 546 phalloidin and examined by confocal microscopy. Merged images are shown on the right. The results illustrated in this figure are representative of five separate experiments.

The PKC inhibitor GF109203X blocks LTD_4- and TPA-induced stress-fibre formation

Phorbol esters such as 12-O tetradecanoylphorbol 13 acetate (TPA) can directly stimulate classic and novel PKCs, and they stimulate microfilament remodelling in most cells. Fig. 3 shows generation of stress fibres induced in intestinal epithelial cells by 100 nM TPA (15 minutes), and the pattern in this case is similar to that seen in such cells treated with 40 nM LTD_4 for 5 minutes. We have previously established that LTD_4 activates PKCα, βII, δ and ε in these cells (Thodeti et al., 2001). In the present experiments, we used the PKC inhibitors bisindoylmaleimide GF109203X and Gö6976 to evaluate the contribution of different PKC isoforms to LTD_4-induced reorganisation of F-actin in these cells. GF109203X is an inhibitor of both classic and novel PKC isoforms, whereas Gö6976 is a potent inhibitor of classic PKC isoforms (Davies et al., 2000; Martiny-Baron et al., 1993). Pretreatment of the cells with 2 μM GF109203X for 15 minutes, before stimulation with either 40 nM LTD_4 for 5 minutes or 100 nM TPA for 15 minutes, inhibited stress-fibre formation (Fig. 3). By contrast, identical pretreatment with 2 μM Gö6976 did not cause such inhibition (Fig. 3). These results suggest that a novel PKC isoform is involved in the LTD_4-induced rearrangement of the cytoskeleton in epithelial cells.

PKCδ is involved in the stress-fibre formation induced by LTD_4

To further investigate the identity of the PKC isoform(s) involved in LTD_4-induced stress-fibre formation, we transfected cells with vectors encoding the regulatory domain (RD) of PKCα, βII, δ or ε fused to EGFP. These RDs have previously been documented to act as specific dominant-negative inhibitors of their respective PKC isoform (Cai et al., 1997; Jaken, 1996; Kiley et al., 1999; Paruchuri et al., 2002). The results obtained with cells transfected with vectors encoding these RDs revealed that the LTD_4-induced formation of stress fibres was inhibited by the RD of PKCδ but was not affected by the RD of PKCα, βII or ε (Fig. 4).

The activation of PKCδ occurs downstream of RhoA

Considering the above results, we addressed the question of whether the LTD_4-induced activation of PKC-δ occurs upstream or downstream of RhoA. We treated cells with LTD_4 in the presence or absence of the Rho inhibitor C3 exoenzyme and then measured activation of PKCα, δ, and ε by measuring their translocation to the membrane fraction. As outlined in Fig. 5, these three PKC isoforms were rapidly translocated to the membrane fraction upon exposure to 40 nM LTD_4, and the maximum effect of the translocation was seen after 90-300 seconds. However, in the presence of C3 exoenzyme, the translocation to the plasma membrane induced by LTD_4 treatment was reduced for PKCα and ε and was totally inhibited for PKCδ (Fig. 5). In additional experiments, we transfected the cells with N19-RhoA (the dominant-negative of RhoA) or the RD of PKCδ and directly stimulated either PKC with TPA or RhoA with CNF-1 (Fig. 6A,B). Cells transfected with EGFP-N19-RhoA produced stress fibres when exposed to 100 nM TPA for 15 minutes. By contrast, when cells were transfected with the RD of PKCδ, no tendency towards generation of stress fibres was seen after treatment with CNF-1 (Fig. 6B). To confirm these findings, we used another approach to inhibit PKCδ. The cells were stimulated with CNF-1 for 16 hours in the absence or presence of 2 μM GF109203X, and, in agreement, the formation of stress fibres was completely inhibited by GF109203X (Fig. 6C). Together, these results show that activation of PKCδ is needed for stress-fibre formation and that it occurs downstream of RhoA.

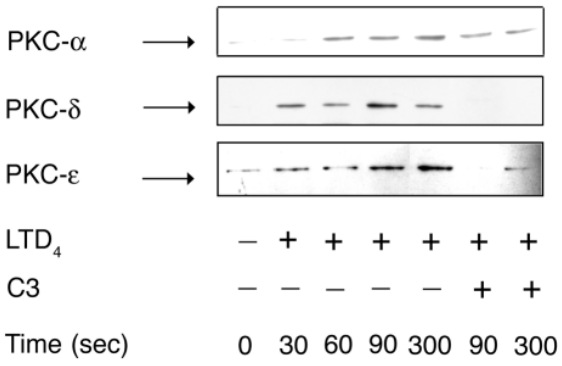

Fig. 5. Effects of C3 exoenzyme on LTD_4-induced translocation of PKCα, PKCδ and PKCε to the membrane fraction. Cells were or were not pretreated with 5 μg/ml C3 exoenzyme together with 5 μg/ml lipofectamine for 10 hours and then were or were not stimulated with 40 nM LTD_4 (30, 60, 90 and 300 minutes). Thereafter, the cells were lysed and centrifuged at 200,000 ***g*** for 30 minutes as described in the Materials and Methods. The resulting membrane-rich pellet was separated by SDS-PAGE and immunoblotted with an anti-PKCα, anti-PKCδ or anti-PKCε antibody. The illustrated blots are representative of at least three separate experiments.

Discussion

The results presented here add to our previous observation that the inflammatory mediator LTD_4 induces changes in the cellular content of F-actin (Massoumi and Sjölander, 1998) by providing evidence that LTD_4 also causes a qualitative rearrangement of the actin cytoskeleton in intestinal epithelial cells by inducing stress-fibre formation. A major finding of our study is that the LTD_4-induced production of stress fibres, which occurs very rapidly, is mediated via an LTD_4-induced activation of RhoA, since both transfection of cells with dominant-negative RhoA (N19-RhoA) and pre-incubation with the potent RhoA inhibitor C3 exoenzyme effectively inhibited the LTD_4-elicited stress-fibre production. In reciprocal experiments, we found that direct activation of Rho by CNF-1 (Flatau et al., 1997; Schmidt et al., 1997) and transfection of cells with a constitutively active RhoA (L63-RhoA) both mimicked the effects of LTD_4. In line with this, it has recently been reported that LTD_4 induces RhoA-mediated actin reorganisation in human bronchial smooth muscle cells (Saegusa et al., 2001). LTD_4 exerts its effect through its G-protein-coupled receptor $CysLT_1$ (Lynch et al., 1999; Thodeti et al., 2000), which interacts with specific targets and thereby initiates a downstream signalling cascade. It has been reported that specific G protein subunits differentially regulate the activation of families of low molecular-weight GTPases, such as Ras and Rho (Boguski and McCormick, 1993; Burgering and Bos, 1995), which may explain the RhoA activation observed in this study. Rho-dependent stress-fibre formation mediated by $G\alpha_{12}$ has been observed in both Swiss 3T3 (Buhl et al., 1995) and SV 40 (Gilchrist et al., 2001) cell lines, whereas Gβγ induces stress-fibre formation in a Rho-dependent manner in HeLa cells (Ueda et al., 2000). We have previously shown that LTD_4 induces formation of a Gβγ/PLC-γ1 signalling complex in intestinal epithelial cells (Thodeti et al., 2000), and more recently we were able to show that this complex contains active RhoA (Thodeti et al., 2002). This finding suggests, but certainly does not prove, that the LTD_4-induced stress-fibre production is mediated through Gβγ-induced activation of RhoA.

LTD_4 can activate different PKC isoforms in Int 407 cells, including PKCα, βII, δ and ε (Thodeti et al., 2001). In addition, we have previously shown that activation of PKCα in LTD_4-treated cells is involved in the formation of focal adhesions and activation of β1 integrins (Massoumi and Sjölander, 2001). In our present investigation, we used two different PKC inhibitors, one for classic isoforms

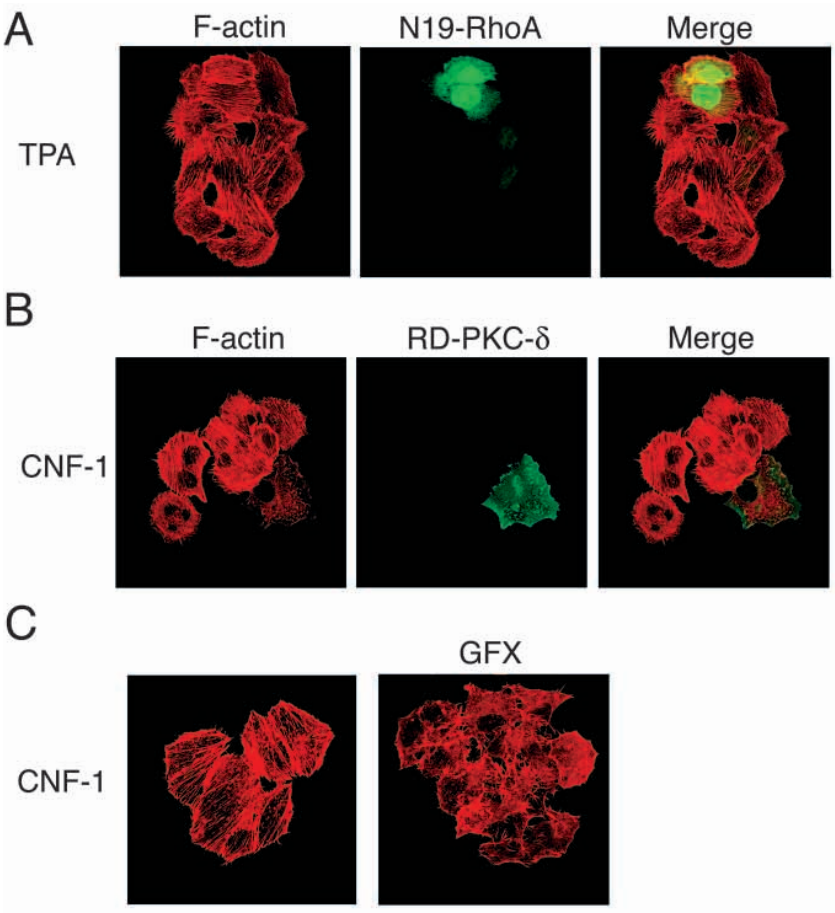

Fig. 6. Effects of RhoA and PKCδ on stress-fibre formation. (A) Cells were transfected with the vector encoding EGFP-N19-RhoA and then stimulated with 100 nM TPA for 15 minutes, and they were subsequently fixed, permeablised, stained for F-actin with Alexa 546 phalloidin and examined by confocal microscopy. (B) Cells were transfected with vector encoding EGFP-RD-PKC-δ and then stimulated with 300 ng/ml CNF-1 for 16 hours and subsequently processed as in A. (C) Cells were stimulated with 300 ng/ml CNF-1 in the absence or presence of 2 μM GF109203X for 16 hours and then processed as in A. Merged images are shown on the right. The results illustrated in this figure are representative of five separate experiments.

(Gö6976) that does not inhibit the LTD_4- or TPA-induced production of stress fibres and one for classic and novel isoforms (GF109203X) that completely inhibits the effects of both LTD_4 and TPA. In addition, we also transfected the cells with vectors encoding the RD of different PKC isoforms to specifically inhibit them (Jaken, 1996) and thereby study the involvement of these enzymes in the LTD_4-induced production of stress fibres. Expression of RD-PKCδ in intestinal cells provided compelling evidence that activation of PKCδ is required for LTD_4-induced stress-fibre formation. It has previously been shown that PKC activation induces or enhances the formation of stress fibres in Chinese hamster ovary cells and fibroblasts (Defilippi et al., 1997; Woods and Couchman, 1992) but not in Swiss 3T3 fibroblasts (Ridley and Hall, 1994). These results and our present observations indicate that PKC activation has cell-type-specific effects on the generation of stress fibres. These specific effects may be caused by altered PKC expression or dissimilarities in PKC-mediated signalling pathways among different cell types. Taken together, our findings suggest that the novel PKC isoform PKCδ is important for actin reorganisation into stress fibres in LTD_4-stimulated Int 407 cells. In contrast to our data, Brandt and co-workers have recently shown that prolonged TPA-induced activation of PKC leads to a disassembly of actin stress fibres after 2 hours by decreasing the activity of RhoA in the rat aortic smooth muscle cell line A7r5 cells (Brandt et al., 2002). It should be pointed out that the cell type used in this study had a well developed actin stress-fibre network that crossed the whole cell body in the resting state, that is, prior to stimulation TPA. The difference between our findings could therefore be ascribed to the cell types used and/or the time of agonist exposure.

We performed several different kinds of analysis to explore a possible relationship between the roles of RhoA and PKCδ in LTD_4-induced stress-fibre formation. First, we could clearly document that the LTD_4-induced translocation of PKCδ to a membrane fraction was blocked by pretreating the cells with the RhoA inhibitor C3 exoenzyme. Thereafter, we examined whether direct activation of PKCδ was sufficient to produce stress fibres when RhoA is inhibited. The results in this case showed that such activation induced by TPA did indeed cause stress-fibre formation in intestinal epithelial cells, even after transfection with dominant-negative RhoA (N19-RhoA). In another attempt to understand the interrelationship between RhoA and PKCδ, we activated RhoA directly by using CNF-1 and inhibited PKCδ by either transfecting the cells with the regulatory domain of PKCδ or pre-incubating the cells with the PKC inhibitor GF109203X. The results revealed that cells with CNF-1-activated RhoA could not produce stress fibres when PKC-δ was inhibited. Together, these data indicate that PKCδ and RhoA are located on the same LTD_4-triggered signalling pathway and that involvement of PKCδ in the present production of stress fibres occurs downstream of RhoA activation. This idea is compatible with our previous observation that inhibition of RhoA impairs the LTD_4-induced and PLC-dependent mobilisation of intracellular Ca^{2+} (Grönroos et al., 1996), since activation of PLC also leads to the formation of diacylglycerol, an endogenous activator of PKC. In contrast to our data, Strassheim and co-workers showed that RhoA and the classic PKCs are involved in separate signalling pathways that regulate the organisation of myosin and stress fibres, and, according to their model of M_3 receptor activation, PKC-induced actin reorganisation does not depend on RhoA activation in a CHO-K1 cell line (Strassheim et al., 1999). However, several studies have demonstrated a convergence between PKC- and Rho GTPase-regulated signalling pathways (Coghlan et al., 2000; Hippenstiel et al., 1998; Nozu et al., 1999). More specifically, Slater and co-workers found that RhoA acts in close association with PKC-α in an in vitro assay (Slater, 2001). A direct interaction between Rho and PKC has also been observed in a study of yeast extracts in vitro, in which Pkc1 co-immunoprecipitated with Rho1 in a GTP-dependent manner (Kamada et al., 1996), perhaps even indicating that Rho1 is located upstream of Pkc1. Interestingly, LTD_4 causes cytoskeleton rearrangements in intestinal epithelial cells by activation of at least two different PKC isoforms. Previous results have shown that LTD_4-induced activation of PKCα affects the formation of focal adhesions and regulation of β1 integrins in intestinal epithelial cells (Massoumi and Sjölander, 2001), whereas the present data show that PKCδ has a specific role in the formation of stress fibres in these cells. Apart from this difference in the dependency on specific PKC isoforms, the formation of stress fibres is a more rapid event, seen already after 5 minutes, in comparison with the formation of focal adhesions, which require 15 minutes exposure to LTD_4. This difference could be explained by the present observation (Fig. 5) that LTD_4 causes a more rapid activation of PKCδ than PKCα does.

We cannot yet define how PKCδ participates in the cytoskeletal rearrangements caused by LTD_4, but interestingly enough Lopez-Lluch and colleaguesrecently reported that the C2-like module, which is located in the N-terminal of PKC-δ, interacts with G-actin in migrating neutrophils and co-localises with F-actin in TPA-stimulated cells (Lopez-Lluch et al., 2001). In order to clarify whether such an event is part of the mechanism whereby PKC-δ participates in LTD_4-induced stress-fibre formation or if PKC-induced phosphorylation of myosin light chain II is involved, as shown for TPA (Masuo et al., 1994), additional experiments have to be performed.

Our results show that activation of the LTD_4 receptor $CysLT_1$ alters the organisation of the actin network by an extensive production of stress fibres in intestinal epithelial cells. This effect is mediated by activation of RhoA and a subsequent downstream activation of the novel PKC isoform PKCδ, both of which are essential for this formation of stress fibres.

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