PKCα-dependent activation of RhoA by syndecan-4 during focal adhesion formation

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Summary

Syndecan-4 is a ubiquitously expressed transmembrane heparan sulphate proteoglycan acting in concert with integrins in the formation of focal adhesions and stress fibres. Signalling events studied thus far suggest the formation of a ternary complex between syndecan-4, phosphatidylinositol 4,5-bisphosphate and protein kinase Cα (PKCα). Syndecan-4 clustering at the cell surface has also been associated with RhoA-dependent signalling, but the relationship between PKCα and RhoA has not been resolved. Here we present evidence that syndecan-4, PKCα and RhoA are in a linear pathway necessary for the formation and maintenance of stress fibres in primary rat embryo fibroblasts. Inhibition of PKCα activity through the use of specific pharmacological inhibitors, a dominant-negative construct, or siRNA downregulation of protein levels, attenuated focal adhesion formation and the maintenance of stress fibres. However, these effects could be bypassed through independent activation of RhoA with lysophosphatidic acid, but not by clustering of syndecan-4 with ligand. Furthermore, inhibition of PKCα could block the increase in the GTP levels of RhoA induced by clustering of syndecan-4 at the cell surface. All these data point to a mechanism whereby syndecan-4 signals to RhoA in a PKCα-dependent manner and PKCα directly influences RhoA activity.

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Key words: Syndecan-4, PKCα, RhoA, Fibronectin, Stress fibres, Focal adhesions

Introduction

Cell adhesion is a complex, multi-stage process during which cells adhering to extracellular matrix (ECM)-coated planar substrates assemble focal contacts with the underlying substratum. For fibroblasts, initial attachment is followed by spreading and the formation of protrusive contacts, termed focal complexes, associated with plasma membrane extensions such as lamellipodia or filopodia. These subsequently mature to form more stable 'focal adhesions' associated with the ends of contractile stress fibres (Adams, 2002). Elucidation of the biochemical composition and the signals that regulate the formation of such adhesion sites and the transition from one type of contact to the other have been a major focus since the identification of integrins, the primary class of ECM adhesion receptors (Miranti and Brugge, 2002). Protein and phospholipid phosphorylation and dephosphorylation events, as well as the activities of small G proteins of the Ras superfamily have been implicated in various stages of cell adhesion, providing a framework of signalling cascades that can regulate cytoskeletal reorganization during cellular interactions with the ECM (DeMali et al., 2003). In particular, Rho family GTPases play instrumental roles in both the formation and the transition from protrusive to contractile adhesions. Hence, following attachment on the ECM protein fibronectin (FN), Rac1 and Cdc42 appear to be activated and contribute to cell spreading through actin polymerization and the formation of focal complexes (Clark et al., 1998; Price et al., 1998; del Pozo et al., 2000). At this stage RhoA activity is suppressed (Ren et al., 1999; Arthur and Burridge, 2001). However, the assembly of stress fibres and subsequent maturation of focal complexes into focal adhesions correlate with, and are dependent on, activation of RhoA signalling (Ren et al., 1999; Rottner et al., 1999).

Syndecans constitute a family of transmembrane proteoglycans involved in the regulation of the actin cytoskeleton by acting as independent receptors or co-receptors with integrins (Couchman, 2003). Of the four mammalian syndecans, only syndecan-4 is detected in focal adhesions in a variety of cells adhering on different ECM molecules including FN (Woods and Couchman, 1994). Plating of cells on the FN fragment of 110 kDa (FN110), which contains the RGD-containing, central cell-binding domain (CBD) of FN, but not the high-affinity heparin-binding region, supports their attachment and spreading by integrin $\alpha_5\beta_1$ but not the formation of focal adhesions (Woods et al., 1986). The complete response is achieved by providing the high-affinity heparin-binding region of FN (HepII) in solution (Woods et al., 1986), or by direct clustering of syndecan-4 with antibodies (Saoncella et al., 1999). Indeed, HepII (Woods et al., 2000) and the isolated FN repeat III_{13} from within the HepII domain (Huang et al., 2001) are ligands for cell-surface syndecan-4. Furthermore, the role of syndecan-4 is confirmed in fibroblasts derived from syndecan-4 knockout mice, where focal adhesions and stress fibres failed to form on FN110 in response to the HepII domain (Ishiguro et al., 2000). Pharmacological studies have also demonstrated that phorbol 12-myristate 13-

acetate (PMA) (Woods and Couchman, 1992) or lysophosphatidic acid (LPA) (Saoncella et al., 1999) can substitute for cell surface stimuli, implicating PKC- and RhoA-dependent pathway(s) downstream of syndecan-4. Furthermore, focal adhesion assembly through syndecan-4 clustering is sensitive to inhibition of RhoA by the C3 transferase (Saoncella et al., 1999), pointing to a requirement for RhoA signalling downstream of syndecan-4.

Signaling events occurring proximal to syndecan-4 include phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] binding resulting in oligomerization of syndecan-4 and the subsequent regulation of PKCα localization and activity through a direct interaction between the catalytic domain of PKCα and the cytoplasmic region of syndecan-4 (reviewed by Couchman, 2003). How RhoA dependent signalling is integrated is unknown. Analysis of the relative activation levels of RhoA during adhesion to FN and FN110 substrata demonstrated that RhoA-GTP levels are increased at a 2-hour time point after seeding on FN (Yoneda et al., 2005) but remained basal on FN110 (A.Y., H. A. B. Multhaupt and J.R.C., unpublished data). These could be elevated following administration of soluble recombinant HepII, implying that syndecan-4 clustering may somehow potentiate RhoA activity (A.Y., H. A. B. Multhaupt and J.R.C.). As syndecan-4 lacks any catalytic activities, its interaction with PKCα might be instrumental in the propagation of signals to the cytoskeleton and Rho family GTPase members. Here it is demonstrated that PKCα and RhoA/Rho kinase (ROCK) are in a linear relationship with PKCα potentially lying upstream owing to its proximity to the receptor. Although both PKCα and RhoA activities are required for focal adhesion formation, inhibition of PKCα through the use of a pharmacological inhibitor, dominant-negative (dn) constructs or small interfering (si) RNA duplexes, can be bypassed by activating RhoA through the LPA–heterotrimeric-G-protein pathway. Furthermore, the syndecan-4 clustering-induced RhoA activation can be inhibited following downregulation of PKCα suggesting that this kinase mediates the activation of RhoA downstream of syndecan-4.
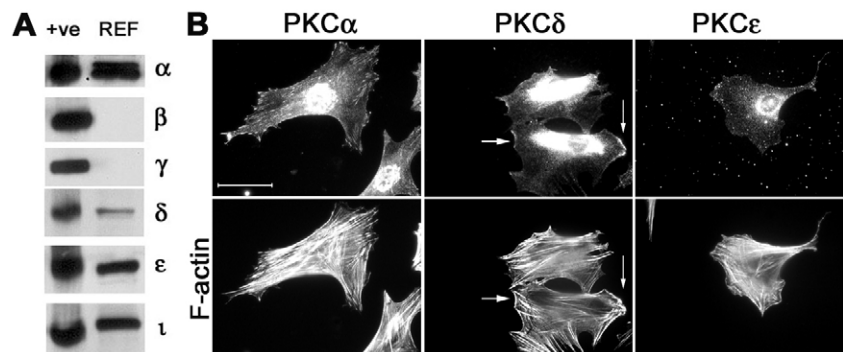
Results

REF cells express PKCα, δ, ε, ζ and ι

PKC constitutes a family of related serine and threonine kinases and includes three subfamilies: the 'conventional' PKCs (α, β and γ), which require diacylglycerol (DAG) and Ca^{2+} for their activation; the 'novel' PKCs (δ, ε, η and θ) which are DAG-dependent but Ca^{2+}-independent; and the 'atypical' PKCs (ι and ζ) for which the mechanisms of activation are still incompletely understood (Mellor and Parker, 1998). Depending on cell type, they can have varying subcellular distributions although the activities of PKCs α, δ and ε have been implicated in integrin-dependent functions (Ivaska et al., 2003) whereas ζ is involved in regulation of polarity during migration (Etienne-Maneville and Hall, 2002). As a first stage to ascertain the role of PKC in stress fibre formation in rat embryo fibroblasts (REFs), PKC expression in these cells was determined (Fig. 1A). Western blot analyses revealed that PKCα was the only conventional isoform expressed. From the novel PKCs, δ and ε were detected, whereas both atypical PKCs, ι and ζ (not shown), were expressed. Immunofluorescence analyses revealed that PKC isoforms α, δ and ε had distinct subcellular distributions, consistent with previous reports (Fig. 1B). PKCα was localized to focal adhesions as previously reported for REF52 and primary REF cells (Jaken et al., 1989; Lim et al., 2003), with juxtanuclear localization also evident. PKCδ had a broad cytoplasmic distribution (Goodnight et al., 1995) with some concentration at the cell periphery, coincident with F-actin in lamellipodia. PKCε staining appeared perinuclear as reported for mouse embryo fibroblasts (Ivaska et al., 2002), with no obvious cytoskeletal association, but consistent with a Golgi-like distribution. These distinct subcellular distributions are suggestive of distinct roles for each PKC isoform.

Inhibition of PKCα interferes with REF stress fibres

To assess the role of PKC isoforms on the cytoskeleton during adhesion to FN substrates, both dnPKC expression and siRNA approaches were used. REFs were co-transfected with plasmids encoding EGFP and dnPKCα. Cells were then cultured in the presence or absence of serum. Serum-free conditions eliminated soluble factors that could influence the cytoskeleton, including LPA. Expression of dnPKCα conferred serum sensitivity of stress fibres to GFP-expressing cells (supplementary material Fig. S1). Although normal REF cells did not lose stress fibres after overnight serum withdrawal, unlike Swiss 3T3 fibroblasts (Ridley and Hall, 1992), PKCα-inhibited cells did so, although cell spreading was not compromised. This effect was reversible, because stress fibres reappeared within 30 minutes of LPA treatment. Consistently, overexpression of dnPKCα-GFP fusion protein potentiated stress fibre disassembly under serum-free conditions, an effect that was reversed upon stimulation of cells with LPA (Fig. 2A). Transfection of a catalytically active

Fig. 1. Expression and intracellular localization of conventional and novel PKC isozymes in REF cells. (A) PKC isoforms expressed in REF cells. REF cell lysates were resolved by SDS-PAGE and probed with monoclonal antibodies against the PKC isoforms indicated. +ve, positive control; REF, REF cell lysate. (B) Differential localization of endogenous PKCs α, δ and ε with respect to the actin cytoskeleton. PKCα is localized at the ends of stress fibres (focal adhesions) and in a juxtanuclear pool, whereas PKCδ is mostly cytoplasmic with some staining associated with cortical actin (arrows). PKCε is perinuclear and shows a Golgi-like staining. Bar, 50 μm.
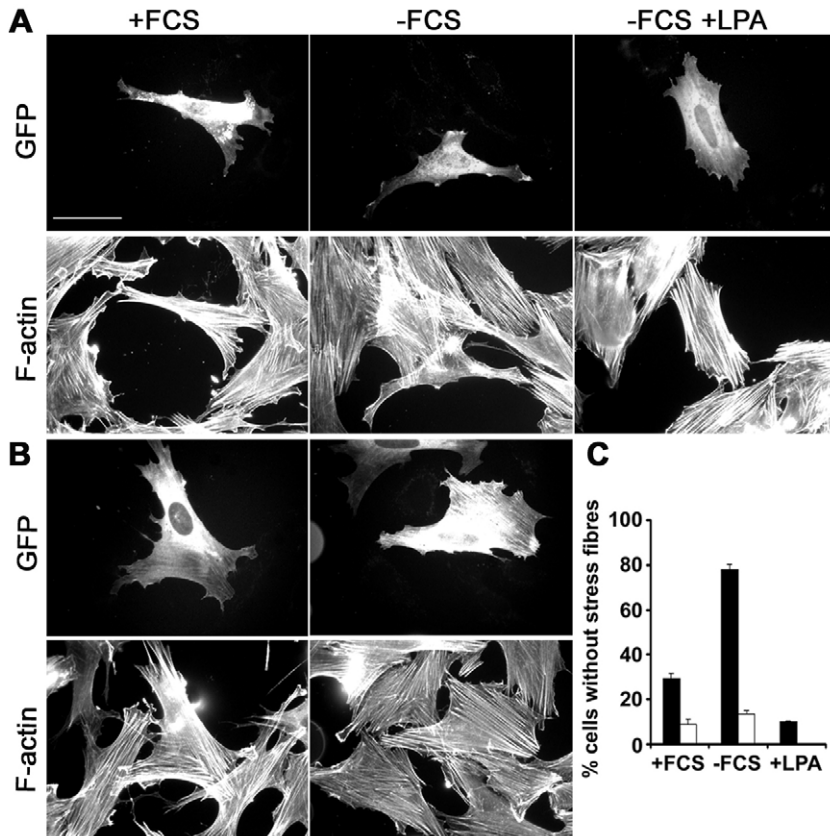
Fig. 2. Overexpression of dominant-negative PKCα results in stress fibre disassembly under serum-free conditions. (A) REF cells were transfected with a plasmid encoding dnPKCα-GFP fusion protein. 24 hours after transfection, cells were either grown with (+FCS) or without (–FCS) serum for a further 16 hours. Some serum-starved cultures were treated for 30 minutes with LPA (400 ng/ml), fixed and stained for F-actin. (B) REF cells were transfected with wtPKCα-GFP and treated as described above. Transfected cells retain stress fibres in the presence or absence of serum. Bar, 50 μm. (C) Quantification of the percentage of cells expressing the PKCα-GFP constructs undergoing stress fibre disassembly. Black bars, dnPKCα-GFP; white bars, wtPKCα-GFP. LPA stimulation was not performed on cells transfected with the wt construct and, therefore, quantitative results are not shown. Results were obtained from three independent experiments (± s.d.).

PKCα-GFP fusion protein did not result in stress fibre disassembly under either condition (Fig. 2B,C). Likewise, siRNA duplexes against PKCα reduced the amount of the endogenous protein (Fig. 3A), and resulted in disassembly of stress fibres and the removal of α-actinin from focal adhesion sites under serum-free conditions (Fig. 3B) but not in the presence of serum (not shown). LPA treatment restored stress fibres and the focal adhesion localization of α-actinin (Fig. 3B).
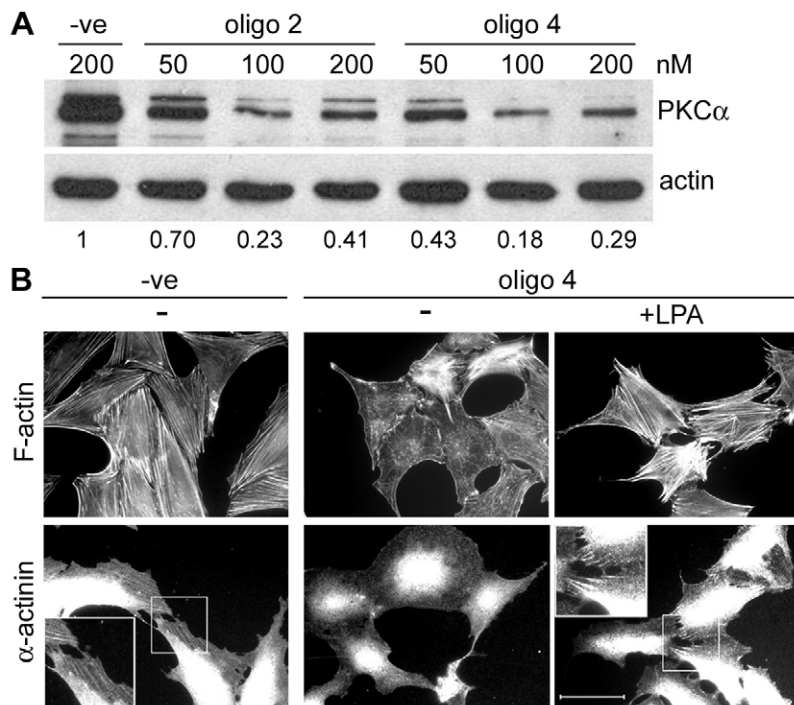
The effect of PKCα inhibition is distinct to that reported for fibroblasts treated with siRNA for ROCK I, which, even in the presence of serum, could not assemble stress fibres (Yoneda et al., 2005). PKCα therefore does not appear to mediate the effects of RhoA on the cytoskeleton in a manner similar to ROCK but rather appears to control earlier events.

Fig. 3. PKCα-deficient REF cells disassemble stress fibres and focal adhesions under serum-free conditions. (A) REF cells were transfected with control siRNA oligonucleotide (–ve) or siRNA oligonucleotides 2 and 4 at the indicated concentrations. 48 hours after transfection, cells were lysed and immunoblotted for PKCα and actin. The amount of PKCα relative to the control, normalized to the actin content, is indicated below each lane. (B) REF cells were transfected with control (–ve) or siRNA duplex 4 (oligo 4) and serum starved for 16 hours. Cells were subsequently left untreated (–) or treated with LPA for 30 minutes (+LPA), fixed and stained for F-actin and α-actinin, as indicated in the Materials and Methods. Boxed areas have been enlarged to highlight the filamentous and focal adhesion localization of α-actinin. Identical results were also observed with oligo 2 (not shown). Bar, 50 μm.

Fig. 4. Rottlerin inhibits cell spreading of REFs on FN but does not interfere with focal adhesions of REFs pre-spread on FN. (A) REF cells were serum starved overnight, trypsinized and either treated with rottlerin at 1 μM (a,b) or 3 μM (c,d) or DMSO (e) in suspension for 20 minutes, then plated on FN-coated coverslips for 2.5 hours. For some cultures, rottlerin-containing medium was replaced with fresh serum-free medium 30 minutes after seeding on the FN substrate to ascertain whether the rottlerin effects were reversible (b,d). Cells were subsequently fixed and stained for F-actin. Bar, 50 μm. The graph (f) shows the cell area (mean ± s.d.) obtained from 40 cells selected at random from a representative experiment. (B) Serum-starved REF cells were spread on FN for 2 hours, then treated with rottlerin at 1 μM (a) or 3 μM (d) for 30 minutes. Cells were fixed, permeabilized and double stained for F-actin (red) and paxillin (green). Arrows indicate cells with a protrusive phenotype, arrowhead indicates a cell with no obvious filamentous actin organization. Bar, 50 μm. The graph (c) depicts the percentage of cells (± s.d.) with stress fibres, protrusive and no F-actin structures under the various rottlerin treatments. Black bars, DMSO; white bars, 1 μM rottlerin; hatched bars, 3 μM rottlerin. At least 30 cells were scored from each of two independent experiments.
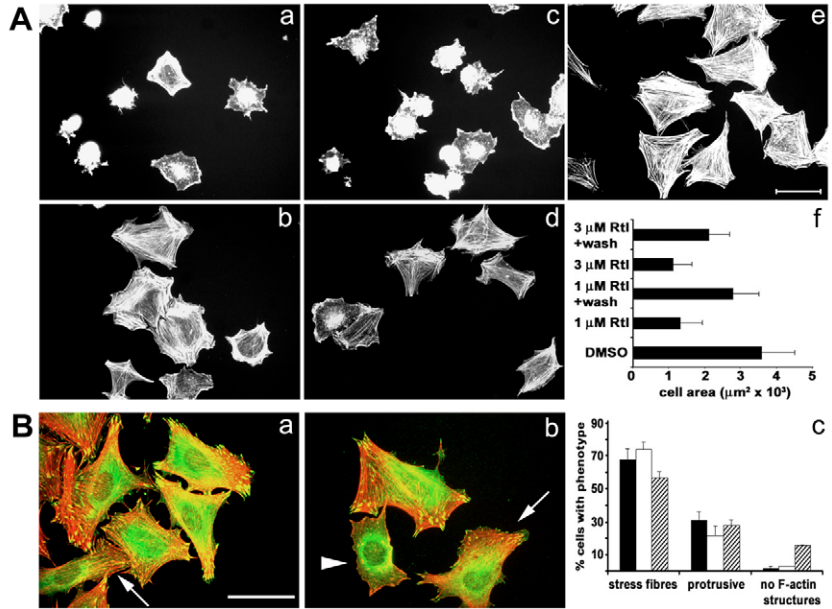
On the other hand, co-transfection of GFP with either the empty vector or with a dnPKCε construct did not affect the actin cytoskeleton whether serum was present or not, whereas dnPKCδ appeared to have severe effects on REFs, causing cell rounding and a complete dissolution of microfilament bundles (supplementary material Fig. S2). As an alternative approach, rottlerin, a specific pharmacological inhibitor of PKCδ activity (IC_{50}=3-6 μM) was used. Administration of rottlerin at concentrations below the IC_{50} value before plating REFs on FN substrata significantly impaired cell spreading (Fig. 4A). This effect was dose-dependent and reversible because replacing the rottlerin-containing media with fresh, serum-free media, restored cell spreading and stress fibre formation. However, administration of rottlerin at 1 μM in REFs already spread on FN did not impair the integrity of focal adhesions and stress fibres (70% assembled stress fibres, 27% had a protrusive phenotype, 3% did not assemble F-actin; comparable with DMSO-treated cells). When administered at 3 μM, although the majority of cells (53%) could assemble focal adhesions and stress fibres, 31% had a more protrusive phenotype, whereas 16% of the cells had no F-actin structures (Fig. 4B). PKCδ is therefore important for the initial spreading response of cells plated on FN, possibly by transducing signals from the integrin receptors.

Inhibition of PKCα activity impairs focal adhesion and stress fibre formation but not cell spreading

The importance of PKCα in cell adhesion was confirmed with the specific PKCα and β inhibitor Gö6976 (IC_{50}=2.3 nM). A dose-dependent reduction in kinase activity was observed with a phospho-specific antibody recognising Thr250 phosphorylation in PKCα (Fig. 5A), a marker of catalytic activity (Ng et al., 1999). On the other hand, phosphorylation levels of the priming sites of PKCα, Thr638 and Ser657 (Newton, 2003), were unaffected by inhibitor treatment

implying that, at the time scale used, the inhibitor downregulated the catalytic activity of the enzyme and did not result in the accumulation of its immature species. Gö6983, a broad-spectrum PKC inhibitor with less potency towards PKCα activity (IC_{50}=7 nM) compared with Gö6976, had a reduced effect on the phosphorylation levels of Thr250 (Fig. 5A) and was therefore not used in subsequent experiments. We note the elevated signal produced by the anti-pT-250 antibody from REF growing under normal conditions. This is consistent with the localization of PKCα in focal adhesions, even in the absence of serum, where its catalytic activity could be required for the maintenance of the cytoskeletal integrity, as determined by the expression of both dnPKCα and siRNA against PKCα. However, the pT-250 antibody appeared not to be suitable for immunofluorescence microscopy and therefore the localization of Thr250-phosphorylated PKCα could not be determined.

Treatment of REF cells with Gö6976 at a range of 0.007-0.25 μM did not impair the formation of stress fibres and focal adhesions of REFs plated on FN. However, treatment at a range between 0.5-1 μM before plating on FN compromised focal adhesion and stress fibre formation but not cell spreading (Fig. 5B). A similar phenotype could be observed after overnight treatment of REFs with a cell-permeable TAT-C3 protein (Sebbagh et al., 2001), which ADP-ribosylates and inhibits RhoA (Fig. 5C), or with the ROCK inhibitor Y-27632 (Yoneda et al., 2005). Therefore, both PKCα and RhoA-ROCK activities are independently required for focal adhesion and stress fibre formation but not the initial attachment and spreading responses.

Administration of Gö6976 (0.5-1 μM) to cells pre-spread on FN also impaired progression from a protrusive to a contractile phenotype. In this case, in the majority of cells (>90%) F-actin was disassembled from the central regions of the cells and focal adhesions were considerably reduced in size (Fig. 6A). PKCα activity appears to be important not only for the maturation of
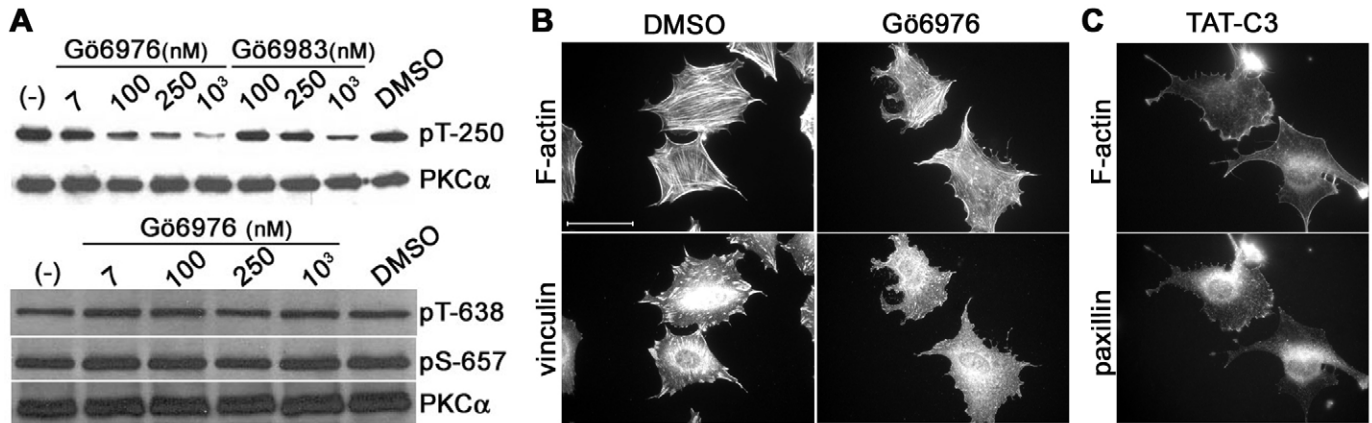
Fig. 5. Pharmacological inhibition of PKCα activity impairs stress fibre and focal adhesion formation but not cell spreading of REFs on FN. (A) REF cells growing under standard conditions were incubated with the indicated amounts of PKC inhibitors for 30 minutes and lysed for western blotting. Equal amounts of lysates were resolved by SDS-PAGE and probed with antibodies against pT-250, pT-638, pS-657 and total PKCα as a loading control, as indicated. (B) Serum-starved REFs were trypsinized and treated with 0.5 μM Gö6976 in suspension for 20 minutes before plating on FN. At 2.5 hours cells were fixed, permeabilized and stained for F-actin and vinculin. (C) REFs were incubated with cell-permeable C3 transferase (TAT-C3) protein (45 μg/ml) in growth medium overnight at 37°C. Trypsinized REFs were replated on FN for 2.5 hours, fixed with 4% paraformaldehyde, permeabilized and stained for F-actin and paxillin. Bar, 50 μm.

focal complexes to focal adhesions but also for the maintenance of focal complexes. LPA, known to activate RhoA, was able to reverse the inhibitory effect of Gö6976 when the latter was administered at either 0.5 μM (Fig. 6A) or 1 μM concentrations (not shown), showing that cells were still competent to form focal adhesions and stress fibres when PKCα activity was downregulated provided that GTP-RhoA levels were elevated.

Although Gö6976 has also been reported to be a rather selective inhibitor of conventional PKCs, it also shows inhibitory activity towards PKCμ/PKD. However, overexpression of either wild-type or dnPKD-GFP (D733A) did not result in disassembly of stress fibres of REF cells grown in the presence or absence of serum (Fig. 6B). A second dnPKD-GFP construct (S744A/S748A) also did not affect stress fibre maintenance (not shown). This indicated that the

effects observed were not due to PKD inhibition. PKD has been shown to be required for the anterograde transport of $\alpha_v\beta_3$ integrin but not $\alpha_5\beta_1$ (Woods et al., 2004), the primary integrin receptor in these FN adhesion assays.

Inhibition of PKCα activity impairs syndecan-4- but not LPA-induced activation of RhoA

Fibroblast adhesion to the integrin-binding FN110 fragment promotes $\alpha_5\beta_1$ integrin-dependent cell attachment and spreading. Under these conditions, cells appear protrusive, with F-actin assembled cortically and numerous focal complexes associated with cellular protrusions. Administration of either LPA or soluble recombinant HepII promoted development of focal adhesions and stress fibres (Fig. 7A). Pre-treatment with Gö6976 alone resulted in disassembly of F-actin and in reduction in the size of focal contacts (Fig. 7A). Under

Fig. 6. Loss of focal adhesions by pharmacological inhibition of PKCα activity can be overcome by activation of RhoA by LPA. (A) REF cells were plated on FN-coated coverslips and allowed to adhere. At 1.5 hours following seeding, DMSO or Gö6976 (0.5 μM) was added. 30 minutes later some of the cells that had received Gö6976 were either left unstimulated or treated with LPA (400 ng/ml) for 30 minutes (Gö6976 +LPA). Cells were fixed, permeabilized and stained for F-actin and vinculin. Bar, 50 μm. (B) REF cells were transfected with plasmids encoding wt or dnPKD-GFP fusion proteins. 24 hours after transfection cells were either kept in normal growth conditions (+FCS) or serum-starved for 16 hours (–FCS). Cells were fixed and stained for F-actin. Bar, 50 μm.
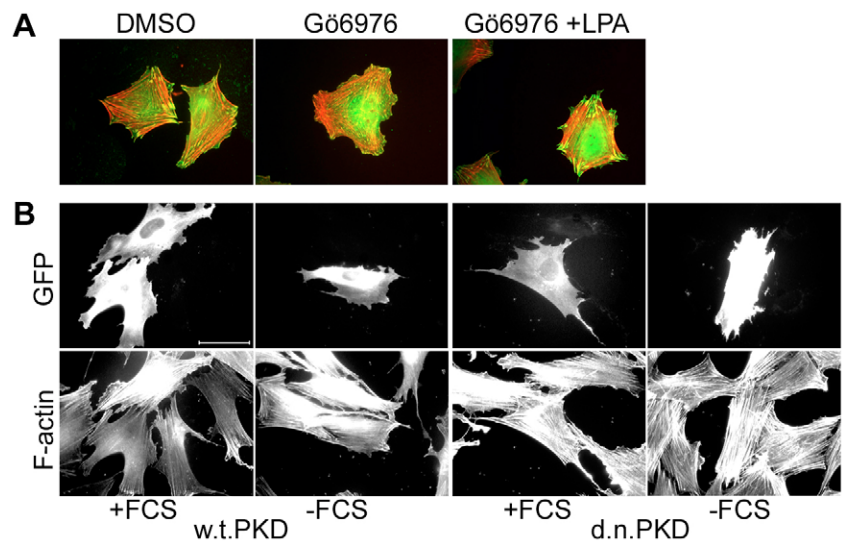
Fig. 7. Lack of focal adhesions resulting from PKCα inhibition can be overcome by LPA treatment but not by HepII and results in recruitment of syndecan-4. (A) REF cells were plated on FN110-coated coverslips for 1.5 hours and either treated with DMSO or with 0.5 µM Gö6976. Cells were subsequently cultured without further treatment (–) or treated with LPA, recombinant HepII, or vehicle as indicated in the Materials and Methods for 30 minutes and cells were fixed, permeabilized and double stained for F-actin (red) and vinculin (green). The percentage of cells forming stress fibres and focal adhesions under the various treatments is indicated. Results are representative of at least three independent experiments. Bar, 50 µm. (B) REFs were plated on FN- or FN110-coated coverslips. At 2 hours, cells on FN110 were either left untreated (–) or treated with LPA, HepII, or pre-treated with Gö6976 for 30 minutes before LPA stimulation, as described above. Cells were subsequently fixed and stained for syndecan-4 and α-actinin. Arrows indicate localization of syndecan-4 and α-actinin to focal adhesions, arrowheads to focal complexes. Bar, 50 µm.
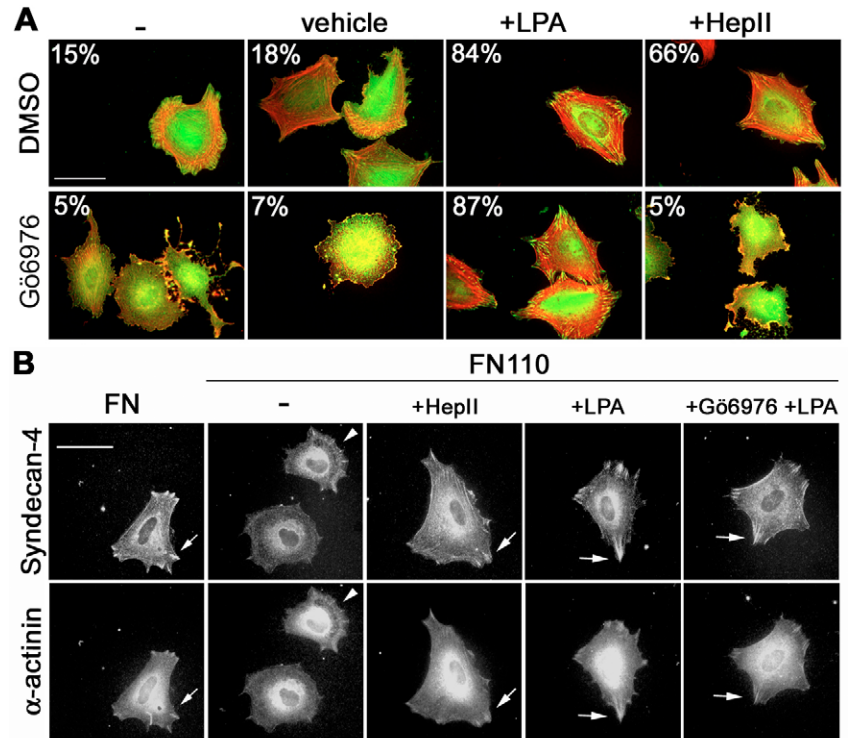
these conditions, soluble HepII could not promote the formation of stress fibres and focal adhesions, unlike LPA, which was sufficient to restore the formation of these structures. Focal adhesion formation in REF pre-spread on FN110 by either HepII or LPA resulted in the same recruitment of focal adhesion components such as vinculin (see Fig. 7A), α-actinin (Fig. 7B), paxillin, or tyrosine-phosphorylated proteins as detected with 4G10 antibody staining (not shown). Syndecan-4 was also recruited to focal adhesions and co-localized with α-actinin following either HepII or LPA treatment. This was unchanged even in cases where PKCα was inhibited by Gö6976 prior to LPA stimulation (Fig. 7B).

To confirm that the effect of LPA treatment depends on RhoA signalling, cells were pre-treated with Gö6976, the ROCK inhibitor Y-27632 or with TAT-C3 to inhibit RhoA directly, and subsequently stimulated with LPA. Stress fibre formation and myosin light chain (MLC)-2 phosphorylation, a major substrate of ROCK I and II (Riento and Ridley, 2003), were assessed. As shown in Fig. 8, treatment with either C3 transferase or the ROCK inhibitor Y-27632 respectively decreased MLC2 phosphorylation, which was no longer localized along stress fibres. However, in Y-27632-treated cells administration of LPA induced cortical actin polymerization, unlike the C3-treated cells, as previously reported (Tsuji et al., 2002). This can be explained by the fact that, in the Y-27632-treated cells, RhoA was still active and able to stimulate its effector mDia and subsequently Rac1, whereas C3, by inhibiting RhoA, prevented any effector activation (Tsuji et al., 2002). This indicated that treatment of cells with LPA, which appears to be a PKCα-independent pathway, can efficiently replace syndecan-4 clustering as the stress fibre and focal adhesion promoting stimulus and depends on RhoA-ROCK signalling.

The inhibitory effect of downregulating PKCα activity on the ability of HepII to stimulate focal adhesion formation

suggested an inhibition or impairment of an increase of RhoA-GTP levels induced by syndecan-4 clustering. This was confirmed by analysis of RhoA-GTP levels in pull-down experiments with a Rhotekin fusion protein (Ren et al., 1999). Under control conditions (Fig. 9A; DMSO), addition of soluble HepII to REFs pre-spread on FN110 substrate induced an increase in the levels of GTP-loaded RhoA, peaking at 10 minutes. Treatment with Gö6976 however, abolished any increase in response to HepII addition, maintaining RhoA activity at basal levels, indicating that PKCα activity was necessary for syndecan-4 to activate RhoA (Fig. 9A). Independent experiments using LPA as a stimulus showed that, even though the pattern and the level of increase in GTP-RhoA levels was different between experiments (see Fig. 9B for a representative example), the level of increase in the GTP load of RhoA was similar between cells that had been pre-treated with either Gö6976 or DMSO within the same experiment (Fig. 9B, inset). Inhibition of PKCα therefore does not prevent RhoA activation by LPA, explaining why it can act as an effective bypass in the formation of focal adhesions and stress fibres. Despite the fact that the relative activation of RhoA-GTP levels appears to be larger following LPA stimulation compared with HepII, the modest increase observed by the latter is sufficient to drive stress fibre and focal adhesion assembly. This appears to be similar to that observed by Ren et al. (Ren et al., 1999), where Swiss 3T3 cell adhesion on FN caused a ~1.5-fold increase in RhoA GTP levels whereas the presence of serum dramatically increased them.

Discussion

Syndecan-4 is a heparan sulphate proteoglycan co-receptor that can respond to adhesive inputs to promote focal adhesion formation and modulate migratory behaviour (Couchman, 2003). It therefore affects integrin-mediated cell-matrix
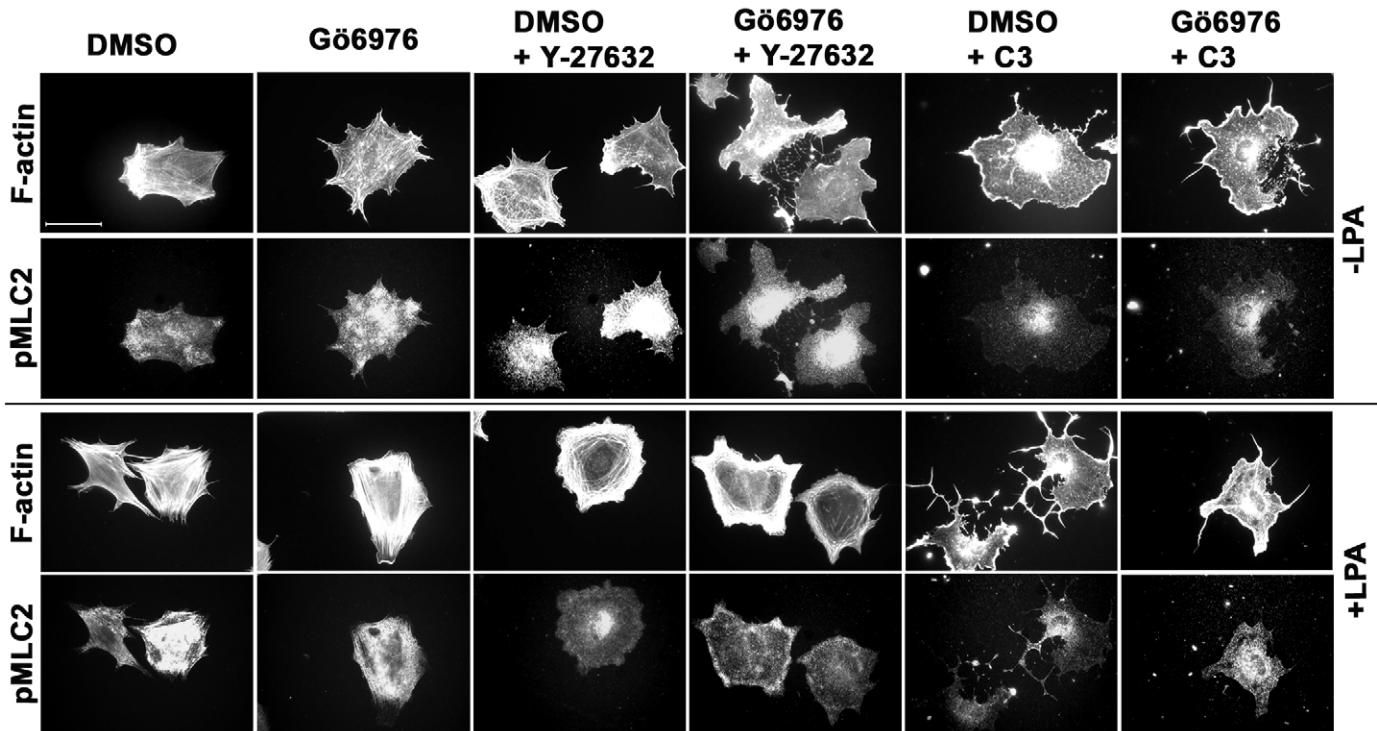
Fig. 8. LPA depends on RhoA-ROCK, not PKCα, in order to phosphorylate myosin light chain. REFs plated on FN110 for 1.5 hours were treated with Gö6976, Y-27632 or pre-treated with TAT-C3 before plating as indicated in the Materials and Methods, followed by stimulation with LPA at 2 hours. After a further 30 minutes, cells were fixed, permeabilized and stained for F-actin and phospho-MLC (pMLC2). Bar, 50 μm.

adhesion and signalling involves unique associations with signalling and cytoskeletal proteins. PKCα is associated with focal adhesions in REF52 and primary REF cells (Jaken et al., 1989; Lim et al., 2003) (this study) and a number of adhesion receptors, cytoskeletal and focal adhesion proteins serve as interaction partners and substrates both in vitro and in vivo thus promoting cytoskeletal rearrangements and influencing migratory behaviour in a variety of cell types (for reviews, see Ivaska et al., 2003; Poole et al., 2004; Larsson, 2006). However, the role of PKCα with respect to syndecan-4 signalling is currently unknown. The interaction of PKCα with syndecan-4 appears to involve a PtdIns(4,5)P_2-rich membrane environment, and results in potentiation of Ca^{2+}-independent

catalytic activity (Couchman, 2003). Direct interaction with the receptor is mediated by the catalytic domain of PKCα (Lim et al., 2003), which may be achieved by prior PKCα activation (Oh et al., 1997). A PtdIns(4,5)P_2-binding region in PKCα resides in a lysine-rich cluster in its C2 domain leading to partial enzyme activation in the absence of Ca^{2+} (Corbalán-

Fig. 9. Inhibition of PKCα prevents activation of RhoA induced by syndecan-4 clustering. REF cells were plated on FN110-coated dishes for 1.5 hours and DMSO or Gö6976 (1 μM) were added for a further 30 minutes. Subsequently, cells were stimulated with recombinant HepII (1 μg/ml; A), or LPA (400 ng/ml; B). Cells were lysed at the indicated time points and GTP-bound RhoA was captured on GST-Rhotekin-RBD-conjugated glutathione-agarose beads. Samples were resolved by SDS-PAGE, immunoblotted for RhoA and the relative activities of the protein at the indicated time points under each condition determined densitometrically. ■, DMSO; ▲, Gö6976. (A) Results were obtained from three independent experiments (mean + s.e.m.; n=3). **P<0.05 compared with levels in Gö6976-treated cells. (B) Results obtained from one of three independent experiments are shown in the graph. The inset shows the ratio of relative RhoA activity between Gö6976- and DMSO-treated cells at various time points following LPA stimulation (mean ± s.e.m.; n=3).
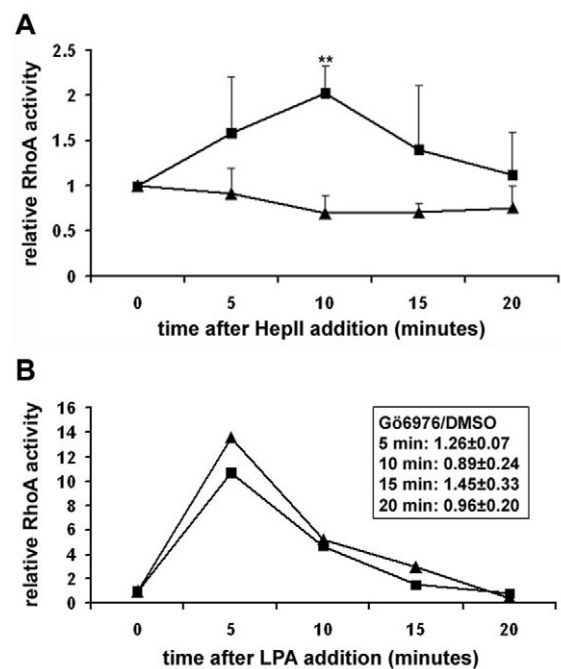
García et al., 2003). This interaction may relieve autoinhibition of PKC and direct it to a syndecan-4-regulated membrane microdomain where it can locally phosphorylate specific substrates. Potential candidates include regulators of the small GTPase RhoA that promotes focal adhesion and stress fibre assembly. RhoA-GTP is required for signalling following syndecan-4 engagement at the cell surface (Saoncella et al., 1999) where increases in GTP load and hence activity are seen (Fig. 9) (A.Y., H. A. B. Multhaupt and J.R.C., unpublished data). One substrate appears to be RhoGDIα, which is known to be regulated by phosphorylation (reviewed by Dovas and Couchman, 2005) (our unpublished observations).

Here we addressed the question of whether the syndecan-4-dependent activation of RhoA is mediated by PKCα, and our data suggest that it does. Inhibition of either PKCα or RhoA can inhibit focal adhesion formation in REFs plated on FN substrates. The data suggest that PKCδ may be involved in the early stages of cell adhesion and influence integrin-mediated cell spreading. However, prolonged inhibition by a dnPKCδ construct may compromise other cellular functions, indirectly affecting the cytoskeleton. A PMA-responsive PKC activity before cell spreading on FN has been proposed (Vuori and Ruoslahti, 1993). On the other hand, PKCε inhibition appears not to affect stress fibre maintenance in REFs though inside-out signalling and delivery of β_1 integrins to the plasma membrane during migration may involve PKCε (Ivaska et al., 2002). This would not be evident under the experimental conditions used here. Cell spreading on FN requires PKCδ activity followed by a switch to PKCα activity in stress fibre and focal adhesion formation.

Although normal REFs maintain stress fibres under serum-free conditions, PKCα-inhibited cells disassembled them but retained a spread phenotype, similarly to cells siRNA-treated to downregulate ROCK I (Yoneda et al., 2005). Under resting conditions, PKCα is localized to focal adhesions, potentially providing signals contributing to their maintenance, even in the absence of serum, which contains LPA, to independently activate RhoA. Downregulation of endogenous PKCα activity may therefore prevent this cytoskeletal maintenance, yet expression of the dnPKCα or siRNAs did not compromise cell viability, because administration of LPA re-established stress fibres. This is distinct to what was observed with ROCK I downregulation, where cells grown in the presence of serum did not assemble stress fibres (Yoneda et al., 2005) and the cumulative evidence therefore places PKCα upstream of the RhoA-ROCK I pathway.

Inhibition of PKCα also affected maturation of focal contacts, but not cell spreading, during the dynamic cytoskeletal rearrangements that take place throughout adhesion on FN. The inhibitory effect of PKCα downregulation could again be efficiently bypassed by LPA treatment of cells, activating RhoA through heterotrimeric G proteins and a tyrosine kinase-dependent mechanism (Kjøller and Hall, 1999). Furthermore, LPA treatment maintained MLC2 phosphorylation in conditions of PKCα but not RhoA or ROCK inhibition, pointing to the dependence of intact RhoA-ROCK, not PKCα, in the LPA effect. However, focal adhesions promoted by LPA recruited the same spectrum of proteins, including syndecan-4, to those promoted by HepII, even under conditions of PKCα inhibition. This is in accordance with published observations that induction of

Tyr397 phosphorylation of FAK in cells plated on FN110 is dependent on RhoA but not PKC signalling (Wilcox-Adelman et al., 2002). However, it also challenges the view that PKC activity is required for the recruitment of syndecan-4 to nascent focal contacts (Baciu and Goetinck, 1995). Even though that study did not distinguish which PKC isoform was responsible, a novel PKC such as PKCδ is unlikely to be mediating this effect; PKCδ phosphorylates syndecan-4 on Ser183 resulting in decreased PtdIns(4,5)P_2 binding, and concomitantly decreased syndecan-4 oligomerization (Couchman, 2003). Localization of syndecan-4 under conditions of PKCα inhibition may be through interactions with α-actinin, which are independent of PKCα, and the binding of these two proteins to syndecan-4 may be mutually exclusive (Greene et al., 2003).

On the other hand, under conditions of PKCα inhibition, syndecan-4 clustering did not promote stress fibres and focal adhesions, indicating that PKCα activity is important for the signalling from syndecan-4. This was substantiated biochemically as RhoA-GTP levels, which were increased by syndecan-4 clustering, remained unaltered when PKCα was inhibited. RhoA-GTP is, therefore, dependent on PKCα activity where adhesion to FN involves syndecan-4.

By contrast, elevated RhoA-GTP was observed in GD25 fibroblastoid cells overexpressing β_1 integrin (GDβ1) when plated on full-length FN or FN110 substrata, even though focal adhesion and stress fibre formation was only evident in those plated on the intact FN. Addition of HepII domain was required for focal adhesion formation in cells plated on FN110 (Danen et al., 2002). Increased RhoA-GTP was possibly a result of β_1 integrin overexpression, because parental GD25 cells did not have comparable RhoA activity levels (Danen et al., 2002). The data suggest a significant role for syndecan-4 nonetheless, perhaps in localizing downstream signalling events to promote cytoskeletal rearrangement.

Wang et al. (Wang et al., 2005) proposed that a recombinant CBD fragment encompassing repeats III$_{8-11}$ was sufficient to promote focal adhesion and stress fibre assembly in a manner that did not correlate with RhoA-GTP levels, in the case of human dermal fibroblasts, or did correlate in mouse FN$^{-/-}$ fibroblasts, pointing to cell type-dependent differences. It was argued that the III$_{8-11}$ fragment fused to GST adsorbed similarly to full-length FN on certain surfaces and it was to adsorption properties that the effect on the FN$^{-/-}$ fibroblasts was attributed. However, these cells cannot form focal adhesions on FN110 (Saoncella et al., 1999), suggesting that FN110 and III$_{8-11}$-GST fusion proteins may possess distinct properties, a question which remains unresolved. It was also proposed that proteoglycan engagement could not support efficient RhoA-dependent signal transduction because cells plated on recombinant HepII exhibited decreased MLC2 phosphorylation after LPA treatment compared with cells plated on recombinant III$_{8-11}$-GST (Wang et al., 2005). We suggest that syndecan-4, a major cell surface receptor for HepII (Woods et al., 2000; Huang et al., 2001), supports both RhoA activation and downstream signalling not independently, but secondary to integrin engagement, which is required for cell spreading before the initiation of contractile activities.

Collectively, the data here suggest a linear pathway from syndecan-4 proceeding through PKCα to RhoA, consistent with the finding that activation of either PKC (Woods and

Couchman, 1992) or RhoA (Saoncella et al., 1999) is sufficient to promote focal adhesion formation in fibroblasts. Other reports have suggested that independent activation of PKC or RhoA is not sufficient to separately promote full adhesion but that both may be needed in distinct pathways, revealing cell-type-dependent differences (Defilippi et al., 1997; Thodeti et al., 2003). It has also been suggested that PKCα activation is downstream of RhoA, facilitating ROCK activation (Barandier et al., 2003) or that RhoA activity is a prerequisite for the membrane translocation and activation of PKCα (Hippenstiel et al., 1998). A further possibility is that PKC and Rho pathways may integrate through a direct interaction (Chang et al., 1998; Slater et al., 2001; Pang and Bitar, 2005) resulting in PKCα activation (Slater et al., 2001). In smooth muscle cells PKCα can contribute to inhibition of myosin light chain phosphatase through phosphorylation of the inhibitory polypeptide CPI-17, independently of RhoA (reviewed by Somlyo and Somlyo, 2003). Our data place PKCα upstream of the RhoA-ROCK pathway in the regulation of actomyosin contractility during cell adhesion, consistent with studies in endothelial barrier dysfunction (Mehta et al., 2001; Holinstat et al., 2003) and neurite retraction (Katoh et al., 1998; Sivasankaran et al., 2004). Although neither has been shown to involve syndecan-4, the regulation of RhoA and hence microfilament contractility by conventional PKCs, is a consistent theme. The mechanisms through which syndecan-4-activated PKCα may regulate RhoA activity in our system are currently under investigation.

Materials and Methods
Antibodies
Monoclonal antibody 150.9 against syndecan-4 ectodomain has been described elsewhere (Oh et al., 1997; Longley et al., 1999). Monoclonal antibody against PKCα (M4) and the polyclonal antibody against PKCα pSer-657 were from Upstate Biotechnology. Monoclonal anti-paxillin antibody (Z035) was from Zymed Laboratories. Monoclonal antibodies against PKCδ (clone 14) and PKCε (clone 21) and the PKC sampler antibody kit were from BD Biosciences. Monoclonal anti-vinculin antibody (hVin1) and rabbit polyclonal anti-α-actinin antibody were from Sigma. Polyclonal antibody against RhoA (119) was from Santa Cruz and polyclonal antibodies against phospho-MLC2 (Ser19) and PKCα pThr-638 were from Cell Signaling Technology. Rabbit polyclonal antibody against PKCα pT-250 was a generous gift from Dr Tony Ng (Kings College London, UK).

Reagents
Rottlerin, Gö6976, Gö6983 and Y-27632 were from Calbiochem. LPA and bovine plasma FN were from Sigma. Human FN110 was obtained from US Biological or derived proteolytically from human plasma FN according to the method by Johansson (Johansson, 1985), giving indistinguishable results.

Plasmids
pGEX-2T-rhotekin RB construct was a kind gift from Alan Hall (University College London, UK). Plasmids encoding dnPKC α, δ and ε were from Shigeo Ohno (Yokohama University, Japan) and have been described elsewhere (Ueda et al., 1996). The plasmid encoding TAT-C3 was from Jacques Bertoglio (INSERM, France) and has been described elsewhere (Sebbagh et al., 2001), and recombinant protein was purified accordingly. Plasmids encoding wild-type (wt) and dn (D733A single point mutant and S744A-S748A double point mutant) PKD were provided by Jim Norman (Beatson Institute, Glasgow, UK) (Woods et al., 2004). pEGFP-N1 was from BD Clontech. cDNA encoding FN repeats III₁₂₋₁₅ (HepII) was from Jean Schwarzbauer (Princeton University, NJ). The expression vector was as described (Woods et al., 2000) and protein was purified on Co^{2+}-charged chelating Sepharose fast flow resin (Amersham). Human wt PKCα cDNA fused to GFP in vector pEGFP-N1 was a kind gift from Christer Larsson (Lund University, Sweden). This was converted to dn form by site-directed mutagenesis at the pseudosubstrate (R22A, A25E) and ATP-binding site (K368R), rendering the kinase in an open conformation and catalytically inactive, respectively, using the following mutagenic primers (where the underlined nucleotides denote the mutated sequence): R22A, A25E forward, GCCGCCAAAGGGGAGCTGAGG and reverse, GAAGCGGTT-GGCCACGTCCTG; K368R forward, GCAATCAGAATCCTGAAGAAGG and reverse, ATACAGTTCTTCTGTGCCCTTC.

Cell adhesion and pull-down assay
10 (LPA) or 15 (HepII) cm cell culture dishes (Corning Costar) were coated with FN110 (5 µg/ml) overnight at room temperature. Coated dishes were blocked with 1 mg/ml heat-denatured BSA in PBS for 1 hour at room temperature and washed three times with PBS. REF cells were maintained in α-MEM (Invitrogen) containing 5% FCS (Labtech International). 80% confluent REF cells were incubated overnight with serum free α-MEM. REFs were trypsinized, resuspended in α-MEM containing 0.25 mg/ml soybean trypsin inhibitor and 0.1 mg/ml BSA and collected. After removal of trypsin inhibitor, REF were suspended in α-MEM containing 0.1 mg/ml BSA and kept in suspension for 30 minutes at 37°C. Cells were plated on FN110-coated dishes and at 1.5 hours they were either treated with 1 µM Gö6976 or DMSO. Recombinant HepII (1 µg/ml) or LPA (400 ng/ml) were added at 2 hours following plating and cells were lysed at the indicated time points. Pull-down assays for GTP-loaded RhoA were performed according to Ren et al. (Ren et al., 1999). The signals from western blotting were analyzed by NIH image version 1.61.

Transfection, adhesion assay and immunofluorescence microscopy
REF (4×10^4 cells) were cultured in six-well plates on 13 mm diameter coverslips overnight and transfected with PKC and pEGFP-N1 plasmids (10:1 w/w ratio) using Lipofectamine™ Transfection Reagent (Invitrogen) as described by the manufacturer. Cells were left to recover for 24 hours, after which they were serum depleted overnight. Cells were subsequently left untreated or stimulated with LPA (400 ng/ml) for 30 minutes, fixed with 4% paraformaldehyde in PBS for 15 minutes and stained for F-actin (Alexa Fluor 568-conjugated phalloidin, Molecular Probes). Samples were analyzed on an Olympus Provis AX module fluorescence microscope (Olympus; objectives, UPlanApo 40× 1.0 NA oil iris, UPlanApo 60× 1.4 NA oil; images were collected using a SPOT Insight Mono digital camera) and images were processed using Adobe Photoshop 7.0.

For adhesion experiments, overnight serum-starved REF cells were plated on FN (10 µg/ml; Sigma) or FN110 (5 µg/ml)-coated coverslips and rottlerin, Gö6976 (0.5 µM), Y-27632 (10 µM), LPA (400 ng/ml), TAT-C3 (45 µg/ml), or HepII (1 µg/ml) were added at the indicated times. Cells were fixed with 4% paraformaldehyde in PBS for 15 minutes and permeabilized with 0.1% Triton-X 100 for 10 minutes. Double fluorescence microscopy was performed using the described antibodies, followed by incubation with appropriate fluorochrome-conjugated secondary antibodies and phalloidin (Alexa Fluor 488/568, Molecular Probes). For staining with antibody 150.9 against syndecan-4, cells were fixed in 100% methanol at –20°C, for 20 minutes.

SDS-PAGE and western blotting
REF cells were lysed with 1× Laemmli sample buffer and denatured. Samples were resolved by SDS-PAGE, and transferred onto PVDF membranes (Bio-Rad), blocked in 5% non-fat milk solution in TBS and probed with the indicated primary antibodies followed by incubation with HRP-conjugated secondary antibodies (DAKO Cytomation). For phosphospecific antibody detection, blocking and primary antibody probing were performed in 3% BSA solution in TBS. Detection was performed with an enhanced chemiluminescence detection kit (Amersham Pharmacia).

Silencing of PKCα
Pre-designed sense and antisense siRNA oligonucleotides corresponding to the target sequence for rat PKCα were obtained from Qiagen. The target sequences used were Rn_Prkca_2_HP (referred to as oligo 2) and Rn_Prkca_4_HP (oligo 4). Transfection of siRNA was performed using Oligofectamine reagent (Invitrogen). Optimal silencing was observed 48 hours post-transfection at 100 nM for both oligos 2 and 4 as determined by western blotting and these conditions were used for subsequent phenotypic analyses. A luciferase sequence was used for negative control (100-200 nM), as described (Yoneda et al., 2005). For immmunofluorescence microscopy, cells were either fixed in 100% methanol at –20°C, for 5 minutes and stained for α-actinin, or with 4% paraformaldehyde and stained for F-actin as described above.

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