PDE4-regulated cAMP degradation controls the assembly of integrin-dependent actin adhesion structures and REF52 cell migration

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Summary

Plating of REF52 cells onto extracellular matrix components leads to the formation of integrin-dependent actin adhesion microspikes. We show that the formation of these structures is sensitive to chemical agents that regulate cAMP levels, such as forskolin and IBMX. In particular, by using the specific inhibitor rolipram, we identify the PDE4 family of cAMP-specific phosphodiesterases as critical regulators of this process. The effect of PDE4 on microspike formation is mediated by actions exerted through the activation of PKA – rather than through the alternative cAMP effector, Epac. We provide evidence that peripheral microspikes are RhoA-, ROCK- and myosin-dependent, and that this pathway is suppressed by PDE4 inhibition. In addition, PDE4 inhibition impairs cell locomotion that requires dynamic protrusion and retraction of peripheral spike structures. Our data demonstrate that PDE4 activity is a key modulator of integrin-induced actin assembly at the cell periphery which, in turn, controls cell migration.

Movies available online

Key words: Phosphodiesterase, Rolipram, Actin, RhoA

Introduction

Integrin engagement generates cellular signals leading to the recruitment of structural and signalling molecules that, together with rearrangements of the actin cytoskeleton, leads to the formation of focal adhesion complexes. How these complexes are assembled in response to extracellular matrix (ECM) attachment, and the relationship between early integrin-induced signalling events (the so-called 'inside out signalling') and actin remodelling that promotes focal adhesion formation, are poorly understood (Sastry and Burridge, 2000).

The organisation of peripheral actin and adhesion structures in response to intracellular stimuli requires the correct spatial and temporal organisation of signalling proteins that modulate cellular actin structures. The Rho family of small GTPases comprises the principal effectors of cellular actin remodelling, with RhoA, Rac1 and Cdc42 inducing distinct and well characterised actin structures (reviewed in Hall, 1998). For example, Rac1 and Cdc42 act together at the leading edges of cells to coordinate the formation of lamellipodial and filopodial extensions, by linking the cellular machinery that controls actin nucleation and polymerisation to upstream signals (Machesky and Hall, 1997). Specifically, the localised formation and retraction of leading-edge-actin structures specified by the Rho GTPases controls the traction forces that enable cells to move forward in response to directional cues from the environment. Rho GTPases also promote the assembly of integrin-dependent adhesion complexes that are associated with newly formed actin filaments (Nobes and Hall, 1995).

Cyclic AMP (cAMP) serves as a second messenger that controls many cellular processes (Beavo and Brunton, 2002). Intracellular cAMP can – in some cellular contexts – influence cell shape, although the molecular events underpinning this are not known (Lamb et al., 1988; Lampugnani et al., 1990; Glass et al., 1993). Multiple genes encode a diverse range of phosphodiesterase (PDE) isoforms, which provide the sole means of degrading cAMP within the cell (Manganiello and Degerman, 1999; Francis et al., 2001; Beavo and Brunton, 2002; Conti et al., 2003; Houslay and Adams, 2003). As such, PDEs are able to regulate a diverse range of cellular processes that depend on spatially and temporally controlled intracellular cAMP. Of these, the cAMP-specific PDE3 and PDE4 phosphodiesterases invariably provide the major fraction of cAMP hydrolysing activity in cells. PDE4 enzymes (reviewed in Conti et al., 2003; Houslay and Adams, 2003; Houslay et al., 1998) have recently attracted much interest, as PDE4 selective inhibitors, with rolipram being the archetypal example, appear to have both anti-inflammatory and anti-depressant actions and are currently being developed as potential therapeutic agents for treating major diseases such as asthma, chronic obstructive pulmonary disease (COPD) and rheumatoid arthritis (Spina et al., 1998; Torphy et al., 1998; Giembycz, 2000). PDE4 inhibitors suppress the production of TNF-α, the generation of reactive oxide and the migration of eosinophils and neutrophils which characterise inflammatory lung disease (Spina et al., 1998; Torphy et al., 1998; Giembycz, 2000). In addition, the PDE4D gene has recently been associated with an increased susceptibility to ischemic stroke (Gretarsdottir et al., 2003).

The modulation of cellular morphology by cAMP levels (for

examples see Lamb et al., 1998; Lampugnani et al., 1990; Glass et al., 1993) led us to test whether, and how, PDE activities might contribute to the formation of integrin-dependent adhesive structures. We used rat embryo fibroblasts (REF52 cells), a cell line that has been shown to rapidly spread after attachment to ECM-coated substrata, giving rise to flat cells with distinctive protrusive actin adhesion structures or microspikes (Fincham et al., 2000). We found that the PDE4 family in particular, was required for assembly of the prominent actin adhesion microspike structures that form after integrin engagement. Our data demonstrate that control of cAMP degradation via the PDE4 family is required to regulate cellular responses downstream of integrin engagement, including peripheral actin filament assembly and cell migration.

Materials and Methods

Materials

Antibodies against the following proteins were used: actin, vinculin (Sigma, Poole, UK); scar/WAVE (Upstate, Lake Placid, USA); CREB, phospho-CREB (Ser133-*P*), phospho-MLC (Ser19-*P*) (Cell Signalling Technology, Beverly, USA); protein kinase inhibitor α (PKIα), RhoA, myosin light chain (MLC) (Santa Cruz Biotechnology, USA); Rac1, Cdc42 (all BD Transduction Laboratories, Oxford, UK) and VASP (Calbiochem, San Diego, USA). The following reagents were used at concentrations indicated: forskolin (50 μM), 3-isobutyl-1-methylxanthine (IBMX) (100 μM), rolipram (10 μM), LPA (20 μg/ml) (all Sigma, Poole, UK); Ro-17-2420 (5 μM), cilostamide (1 μM), 8-Br-cAMP (300 μM), H89 (1 μM) (all Calbiochem, San Diego, USA); 8CPT-2Me-cAMP (300 μM) (Biolog, Bremen, Germany); C3 exoenzyme (10 μg/ml in media for 24 hours) (Cytoskeleton, Denver, USA) and Y27632 (10 μM) (Welfide Corporation, Japan).

Cell culture and plasmids

The rat embryo fibroblast cell line REF52 was grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) in 5% CO_2. Cells were plated onto ECM, tissue culture dishes or chamber slides which had been incubated overnight with 10 μg/ml laminin or poly-L-lysine (Sigma, Poole, UK) at 4°C and had been washed extensively with phosphate buffered saline (PBS) before use. Cells were plated in serum free DMEM. Twenty minutes after plating, when cells had adhered but not spread, carrier (usually 0.1% DMSO) or inhibitors were added. Cells were then incubated for a further 40 minutes before fixation or lysis in the appropriate buffer. DNA plasmids were introduced into REF52 cells using Polyfect (Qiagen, Crawley, UK); cells were used 24 hours after transfection. N19RhoA, V14RhoA, V12 Cdc42, N17Cdc42, L61 Rac and N17Rac – all in the pRK5myc plasmid – were kindly provided by Alan Hall (UCL, London, UK). The PKIα construct was a gift from Robert Newton (NHLI, Imperial College, London, UK). The Ht31 construct was a gift from Enno Klussmann (FMP, Berlin, Germany).

Immunofluorescence

Cells were fixed with 5% paraformaldehyde, washed with PBS supplemented with 100 mM glycine, then permeabilised with PBS supplemented with 0.1% saponin and 20 mM glycine. After blocking with PBS supplemented with 0.1% saponin and 10% FCS for 1 hour at room temperature, cells were incubated with primary antibodies at the following dilutions: vinculin, 1:100; myc, 1:100; phospho-MLC (Ser19-*P*), 1:100; PKIα, 1:100; scar/WAVE, 1:400. Primary antibodies were visualised with species-specific fluorescein isothiocyanate (FITC)-coupled or Cy-5-coupled secondary antibodies (dilution 1:100; Sigma, Poole, UK). The localisation of vinculin and of the scar/WAVE isoform in REF52 cells, Scar1, was found to be indistinguishable in these experiments, so antibodies against these proteins were used interchangeably. Actin filaments were visualised with tetramethylrhodamine β-isothiocyanate (TRITC)-phalloidin (1 μg/ml for 45 minutes) (Sigma, Poole, UK). Cells were visualised using a confocal microscope (Leica SP2).

PDE assay

PDE assays were done by a modification (Marchmont and Houslay, 1980) of the two-step method by Thomson and Appleman (Thomson and Appleman, 1971). In brief, cells were lysed in KHEM buffer (50 mM KCl, 50 mM Hepes pH 7.2, 10 mM EGTA. 1.92 mM $MgCl_2$) containing protease inhibitors (Roche Molecular Biochemicals, Germany). Cells were then subjected to a low-speed centrifugation (16,000 ***g*** for 10 min) and aliquots of the resulting supernatant were assayed for PDE activity using 1 μM cAMP, spiked with [^{3}H]cAMP, as a substrate. All assays were carried out at 30°C under conditions where the assay was linear with respect to time and protein concentration. Assays were terminated by boiling, *Crotalus Atrox* venom was added to prevent resynthesis of cAMP and the products of cAMP hydrolyis were separated from unhydrolysed substrate by adding a slurry of Dowex®:H_2O:ethanol (1:1:1). Total PDE activity in cell lysates was determined and is expressed as pmol cAMP hydrolysed per minute per mg of lysate protein. PDE3 and PDE4 activities were determined using the PDE3 selective inhibitor cilostamide (1 μM) (Manganiello et al., 1995) or the PDE4 selective inhibitor rolipram (10 μM) (Houslay et al., 1998).

PKA assay

Cells were lysed in buffer consisting of 50 mM Tris pH 7.5 and 5 mM EDTA supplemented with protease inhibitors. cAMP-dependent protein kinase (PKA) activity was then measured using the peptide substrate Kemptide (50 μM) and reagents from a PKA assay kit (Upstate, Lake Placid, USA). PKA activity was measured in the presence or absence of 20 μM cAMP to determine the percentage of total PKA active under each condition. Non-specific phosphorylation of Kemptide was determined using the PKA inhibitory peptide, PKI (1 μM), and subtracted from total PKA activity.

Western blotting

Cells were lysed as described previously (Fincham et al., 2000). To detect proteins, lysates were subjected to SDS-PAGE, transferred to nitrocellulose and probed with the appropriate antibody at the following dilutions: actin, 1:1000, phospho-CREB (Ser133), 1:1000; total CREB, 1:1000; RhoA, 1:100; Cdc42, 1:250; Rac1, 1:1000; phospho-MLC (Ser19), 1:1000; total MLC, 1:100; VASP, 1:1000 and vinculin, 1:1000.

Rho GTPase assays

GTPase activity assays were performed by the method of Sander and co-workers (Sander et al., 1998; Sander et al., 1999). In brief, REF52 cells were plated onto laminin in the presence or absence of rolipram for 1 hour before being lysed. Lysates from these cells were incubated with the bacterially produced fusion proteins, GST-C21 (to bind GTP-RhoA) or GST-PAK-CD (to bind GTP-Cdc42 or GTP-Rac1), bound to glutathione-coupled agarose beads. The beads were washed in lysis buffer, eluted in SDS-PAGE sample buffer and the amount of bound RhoA, Rac1 or Cdc42 determined by western blotting.

Timelapse video microscopy

Cells were plated on to glass-bottom microwell dishes (Mattek, Ashland, USA) that had been coated with laminin. Twenty-four hours

later cells were transferred to serum free DMEM, and carrier (0.1% DMSO) or rolipram (10 μM) was added. Images of the cells were aquired from a charged-couple-device camera and captured by Open Lab (Improvision Software, UK) every 5 minutes over a 6-hour period. The distance travelled by each cell at each timepoint was calculated based on the change in pixel coordinates of stable structures that were visible within the nucleus. This allowed the calculation of the average speed of each cell. For each experimental condition average cell speed and *P* value were calculated from data obtained from five independent experiments.

Results

Inhibition of PDE4 suppresses the formation of peripheral actin adhesion structures

At early times after plating onto laminin (or fibronectin), REF52 cells spread with radial symmetry. This is accompanied by formation of a dense cortical actin network (shown in Fig. 1A, broken arrow) and prominent microspike structures that contain polymerised actin and adhesion proteins (structures stained with both phalloidin and anti-vinculin) (Fig. 1A, solid arrows). However, when REF52 cells were plated onto poly-L-lysine (which does not support integrin engagement) neither the dense cortical actin nor the actin adhesion microspikes formed at the cell periphery (Fig. 1A, inset). This suggested that assembly of these structures was integrin-dependent. Treatment of REF52 cells with 50 μM forskolin, the direct activator of adenylyl cyclase, prevented the formation of these prominent actin adhesion structures. Instead, the cells exhibited a flattened morphology and extensive lamellipodia/membrane ruffles (Fig. 1B, arrows). A similar effect to that produced by forskolin was observed when cells were plated in the presence of the non-specific PDE inhibitor IBMX (100 μM) (Fig. 1C). These data show that both direct activation of adenylyl cyclase by forskolin and inhibition of cAMP degradation by IBMX, block the formation of actin adhesion microspikes, and lead instead to the formation of large lamellipodia.

IBMX is a non-selective inhibitor as it inhibits multiple PDE family members (Beavo and Brunton, 2002). PDE3 and PDE4 cAMP phosphodiesterases provide the major cAMP degradative activity in many cell types and are both inhibited by IBMX (Beavo and

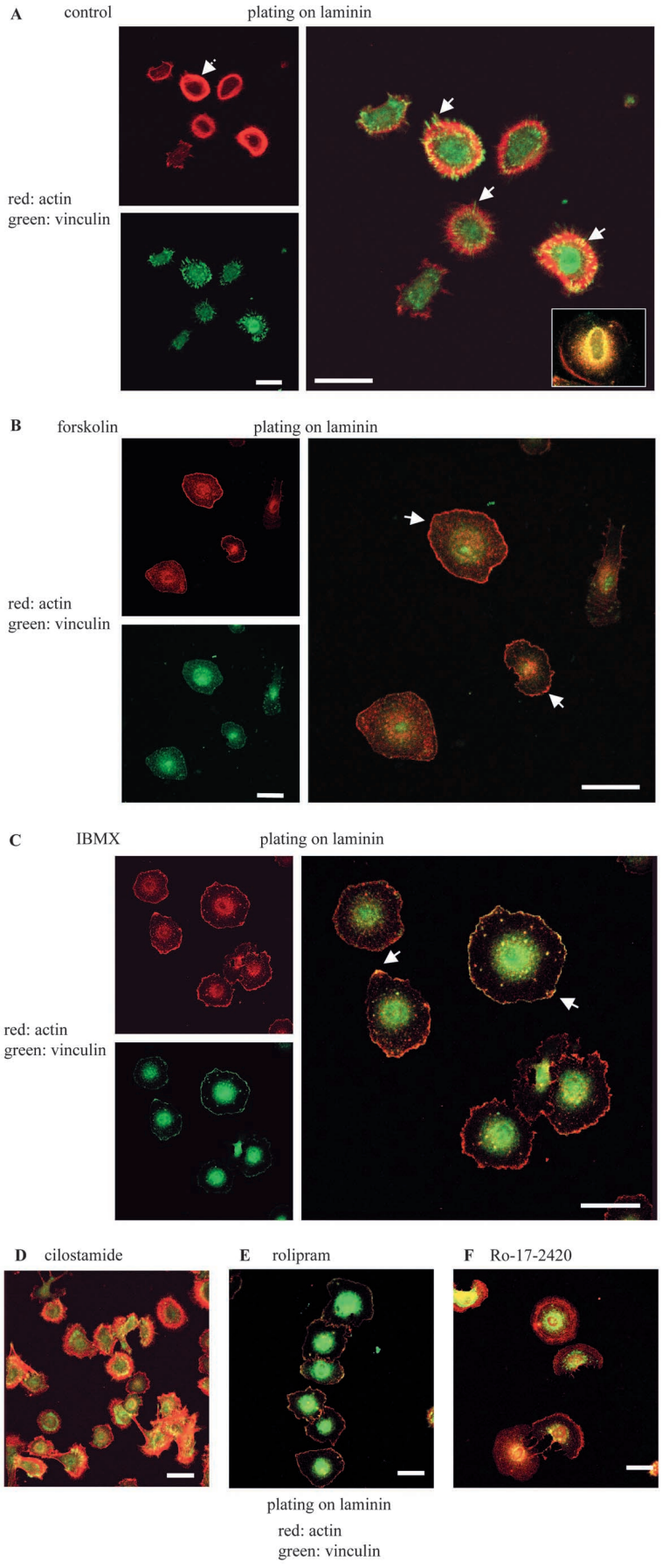


Fig. 1. Integrin-induced actin adhesion microspikes are suppressed by PDE4 inhibitors. REF52 cells were trypsinised, washed in serum free media and allowed to adhere to chamber slides that had been coated with laminin (control conditions) in the presence of (A) 0.1% DMSO carrier, (B) 50 μM forskolin, (C) 100 μM IBMX, (D) 1 μM cilostamide, (E) 10 μM rolipram or (F) 5 μM Ro-172420. Cells were fixed 1 hour after plating and stained for vinculin (green) and with TRITC-phalloidin to visualise actin filaments (red). (A) Cells exhibit a dense cortical actin network (dotted arrow) and prominent microspike structures that contain polymerised actin and adhesion proteins (solid arrows). (B,C) Cells exhibit a flattened morphology and extensive lamellipodia/membrane ruffles (arrows). Scale bars, 40 μm.

Table 1. PDE activities in REF52 cell lysates

Inhibitor present in assay	PDE activity in lysate (pmol/min/mg lysate protein)	Inhibition (%)
None	11.5±0.1	0
Cilostamide (1μM)	8.9±0.8	23±7
Rolipram (10μM)	2.8±0.3	76±3

REF52 cell lysates were prepared and assayed for PDE activity using 1 μM cAMP as substrate. Assays were carried out in the absence of any inhibitor, in the presence of the PDE3 selective inhibitor cilostamide (1 μM) or in the presence of the PDE4 selective inhibitor rolipram (10 μM). Activities are expressed as mean±s.e.m. and were determined in three independent assays.

Brunton, 2002). It is, however, possible to gauge the relative action of these two cAMP phosphodiesterases by chemical 'knockout' using well-defined inhibitors that are selective for enzymes of these two PDE families. Here, we employed both the PDE4 selective inhibitor rolipram and the PDE3 selective inhibitor cilostamide. Used at 10 μM and 1 μM, respectively, actions of these compounds are specific for enzymes of the indicated PDE family and suffice to completely inhibit either PDE4 or PDE3 activity, assessed with 1μM cAMP as substrate (Houslay et al., 1998; Manganiello et al., 1995). Use of inhibitors selective for PDE3 and PDE4, showed that their activities appear to account for essentially all measurable PDE activity in REF52 lysates, approximately 23% and 76%, respectively, of the total PDE activity (Table 1). Such selective inhibitors were therefore employed to determine which of these PDE activities is important for integrin-dependent microspike formation.

Cilostamide had no effect on peripheral microspike formation (Fig. 1D) despite forming around 23% of the total of PDE activity in REF52 cells. However, rolipram, like IBMX, inhibited the formation of actin adhesion microspikes, with cells having a flattened morphology with extensive lamellipodia (Fig. 1E). The structurally unrelated PDE4 selective inhibitor, Ro-17-2420 (5 μM) had an effect that was indistinguishable from rolipram (Fig. 1F). These results suggest that PDE4 activity is important for the assembly of the dense cortical actin that gives rise to the peripheral actin adhesion microspikes, resulting in the distinctive morphology as REF52 cells attach and spread. This identifies a novel mode of controlling the assembly of specific actin structures at the cell periphery via regulated cAMP degradation.

Inhibition of PDE4s leads to the activation of PKA

A major effector of cAMP is cAMP-dependent protein kinase A (PKA) (Bauman and Scott, 2002). In protein lysates from control cells plated onto laminin, only 12 ± 5% of total cellular PKA was active. However, when cells were plated in the presence of the PDE4 inhibitor rolipram, this was increased to 38 ± 2% (Fig. 2A; *n*=3). Also, rolipram caused an increase in phosphorylation of the well-characterised PKA substrates CREB and VASP (Fig. 2B,C). By contrast, the PDE3 inhibitor cilostamide (1 μM) did not increase phosphorylation of CREB or VASP (data not shown).

The PKA selective inhibitor H89 prevented the phosphorylation of CREB in cells treated with rolipram, supporting the assumption that this phosphorylation event is PKA-dependent. However, H89 inhibits other protein kinases

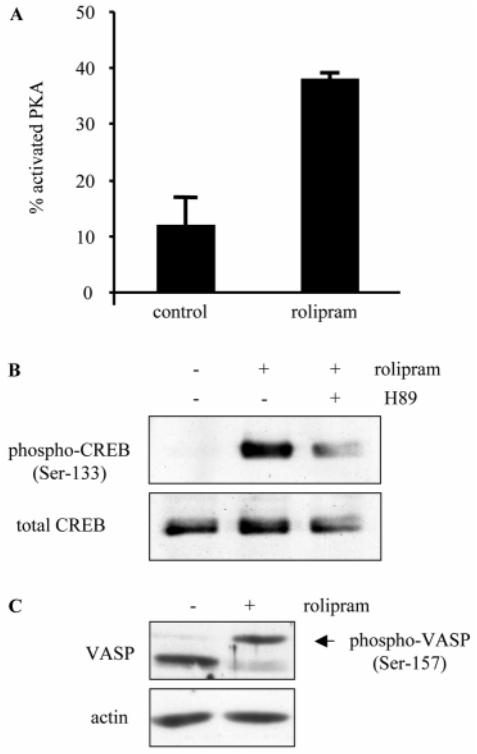


Fig. 2. Rolipram induces protein kinase A activity in REF52 cells. Cells were allowed to adhere to laminin-coated plates in the absence (–) or presence (+) of rolipram before lysis in the appropriate buffer. (A) PKA activity in lysates was assayed using a peptide substrate. Data shown are of three independent experiments (mean±s.e.m.). (B) The level of phosphorylation of the PKA-substrate CREB was determined by western blotting using an antibody specific for CREB phosphorylated at Ser133. An antibody that recognises phosphorylated and unphosphorylated CREB equally well was used to demonstrate equal loading. The PKA selective inhibitor H89 (1 μM) was found to inhibit the rolipram induced phosphorylation of CREB. (C) Western blotting with an antibody that recognises VASP was used to determine the phosphorylation state of this protein as it undergoes a mobility shift when phosphorylated at Ser157. The blot was re-probed with an antibody specific for actin to demonstrate equal loading.

at concentrations similar to those affecting PKA (Davies et al., 2000) and previously observed effects of this inhibitor on cell morphology have been attributed to its inhibition of the Rho kinase ROCK (Leemuis et al., 2002). H89 was therefore unsuitable to determine whether the activation of PKA is required for the loss of peripheral actin adhesion microspikes in cells plated in the presence of rolipram. To circumvent this problem, a construct encoding the physiological PKA inhibitor, PKIα (Day et al., 1989), was used. We found that, after plating onto laminin, cells that expressed PKIα were no longer sensitive to the effect of rolipram (Fig. 3A). PKIα transfected cells exhibited a dense cortical-actin meshwork and similar microspikes to those seen under control conditions (Fig. 3A). This is different in untransfected cells, where rolipram caused the formation of large lamellipodia/membrane ruffles instead, as observed in previous experiments (Fig. 3A). These data show that, following rolipram treatment, the action of PKA is required

to inhibit the formation of dense cortical actin and peripheral actin adhesion microspikes.

Additional cAMP effectors have recently been described, namely the cAMP-regulated guanine nucleotide exchange factors (cAMP-GEFs), also known as Epac, which serve as GEFs for the small GTPases Rap1 and Rap2 (de Rooij et al., 1998; Kawasaki et al., 1998). These effectors influence cell adhesion, although their mechanisms are still unknown (Bos et al, 2001; Rangarajan et al., 2003). To discriminate between the different cAMP effector pathways we utilised 8-Br-cAMP, a cAMP analogue that activates both Epac and PKA (de Rooj et al., 1998), and 8CPT-2Me-cAMP, a cAMP analogue that only activates Epacs without any concomitant activation of PKA (Enserink et al., 2002). We found that 8-Br-cAMP inhibited microspike formation, with cells exhibiting similar morphologies to those treated with forskolin, IBMX or rolipram (Fig. 3B). By contrast, 8CPT-2Me-cAMP had no effect on actin adhesion microspike formation (Fig. 3B), implying that, following cAMP elevation, it is activation of PKA rather than of the Epac-Rap pathway, which results in the suppression of peripheral actin adhesion microspike assembly.

Over 16 different PDE4 isoforms are generated from four genes through the use of specific promoters and alternative mRNA splicing. Some, but by no means all, of these isoforms are targeted to specific subcellular locations leading to the hypothesis that specifically anchored PDE4 isoforms might control particular processes (Conti et al., 2003; Houslay and Adams, 2003). The processing of localised changes in cAMP concentrations is thought to occur through the action of AKAP- (a kinase anchor protein) anchored PKA (Skalhegg and Tasken, 2000; Diviani and Scott, 2001). It has been shown previously that catalytically inactive variants of PDE4s can act as dominant negatives (DN) when they serve to displace anchored active PDE4 associated with the regulation of anchored PKA (Perry et al., 2002; Baillie et al., 2003). We thus set out to see whether we could reproduce the effects of rolipram by overexpressing various DN-PDE4s (PDE4A5, PDE4A10, PDE4B2, PDE4C2, PDE4D3, PDE4D4 and PDE4D5) in REF52 cells but were without success in doing so (data not shown). The reason might be that we have not identified the 'correct' PDE4 isoform(s) that regulate microspike formation. However, given the fact that Ht31, a peptide that disrupts PKA anchoring to AKAPs (Klussman et al., 1999), also failed to affect microspike formation it might well be that this process is not regulated by anchored PKA but rather that it is controlled by bulk cellular cAMP levels affecting a predominantly soluble pool of PKA. If so, then this process is probably sensitive to changes in bulk PDE4 activity rather than being controlled by a specifically anchored PDE4 isoform, which would be consistent with the lack of effect of dominant negative species that can only exert actions by displacing a specific isoform from a functionally relevant anchor site.

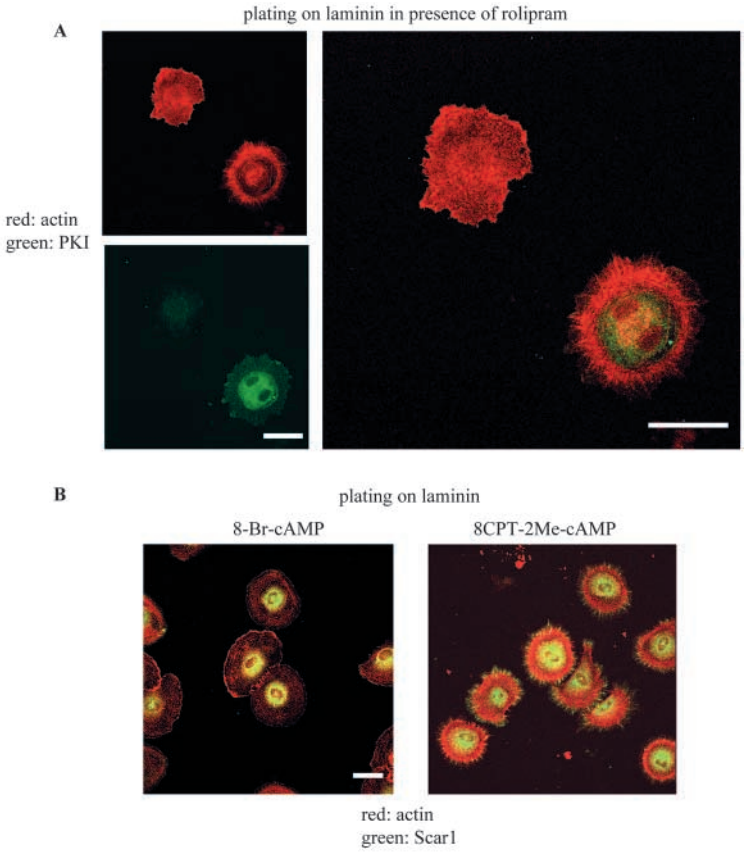
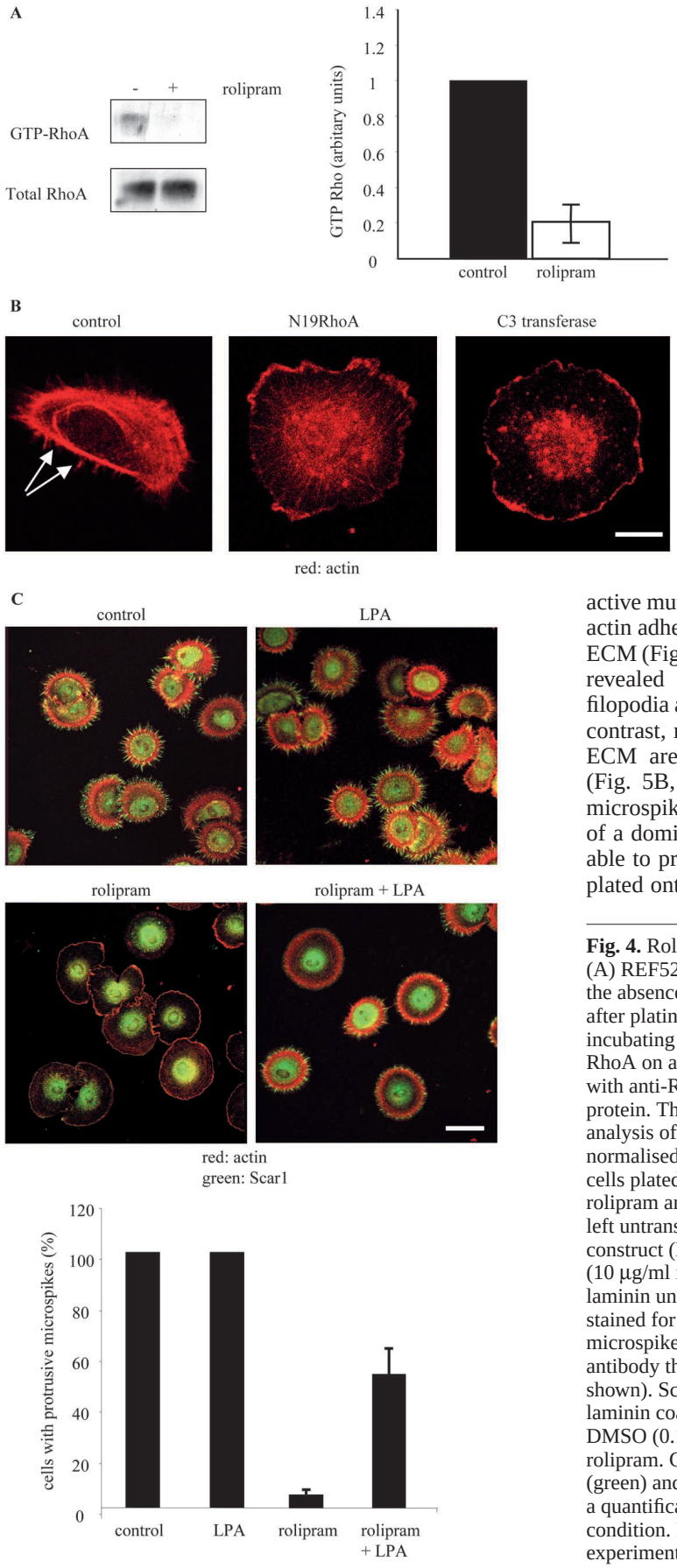


Fig. 3. Inhibition of adhesion spikes by rolipram requires the activation of PKA. (A) Cells were transfected with the protein kinase A inhibitor PKIα before being plated onto laminin in the presence of rolipram. Cells were fixed 1 hour after plating and stained for PKIα (green) and actin (red). (B) Non-transfected cells were plated onto laminin in the presence of 8Br-cAMP or 8CPT-2Me-cAMP (both 300 μM). Cells were fixed 1 hour after plating and stained for Scar1 (green) and actin (red). Scale bars, 40 μm.

Signalling from PDE4 to Rho GTPases

The Rho family of small GTPases, consisting of RhoA, Rac1 and Cdc42, are important effectors of intracellular actin remodelling (reviewed in Hall, 1998). We therefore investigated whether their activities change following the inhibition of PDE4 enzymes and whether this is responsible for the impaired actin remodelling observed. We consistently found a decrease in the level of GTP-RhoA in cells that had been plated onto laminin in the presence of rolipram (Fig. 4A). In addition, formation of integrin-dependent actin microspikes was prevented by expression of a dominant negative mutant of RhoA (N19RhoA), or by treatment of the cells with the RhoA inactivating toxin C3 transferase (Fig. 4B), indicating that the formation of these structures was a RhoA-dependent process. Also, lysophosphatidic acid (LPA), a serum constituent that activates RhoA, rescued the effects of rolipram and restored the assembly of both dense cortical actin and peripheral actin adhesion microspikes (Fig. 4C), demonstrating that RhoA was both necessary and sufficient to induce formation of these structures.

Interestingly, PKA can phosphorylate RhoA and inhibit its activity (Lang et al., 1996; Laudanna et al., 1997; Dong et al., 1998; Tamma et al., 2003; Ellerbroek et al., 2003). The ability of LPA to rescue the negative effects of rolipram on integrin-dependent, RhoA-mediated actin organisation presumably



occurs by a mechanism that either predominates over the PKA-mediated phosphorylation mentioned above or stimulates a different pool of cellular RhoA. However, we have established that LPA is not able to rescue the inhibitory effects of the ROCK inhibitor Y27632 on actin adhesion structure assembly, confirming that LPA is not inducing actin-regulatory events that occur downstream or independently of RhoA (not shown). It is possible however, that LPA, can counteract the effect of rolipram by reducing cAMP levels through the inhibition of adenylyl cyclase activity in intact cells.

It has been shown that Cdc42 and Rac1 are activated in cells plated onto ECM (Price et al., 1998; Ren et al., 1999). Furthermore, PKA has been reported to positively regulate the activities of Cdc42 and Rac1 (Feoktistov et al., 2000; O'Connor et al., 2001). However, in REF52 cells we did not observe any changes in the levels of GTP-Cdc42 or GTP-Rac1 when cells were plated in the presence of rolipram (Fig. 5A,C). Filopodia induced by the expression of a constitutively active mutant of Cdc42 (V12Cdc42) bear some resemblance to actin adhesion microspikes formed after cells were plated onto ECM (Fig. 5B). However, close examination of these structures revealed that they are quite different: the Cdc42-induced filopodia are long, fine and branched (Fig. 5B, arrowheads). By contrast, microspikes that formed after cells were plated onto ECM are short and thick, with no evidence of branching (Fig. 5B, solid arrows). Also, in support of actin adhesion microspikes being regulated differently to filopodia, expression of a dominant inhibitory form of Cdc42 (N17Cdc42) was not able to prevent the formation of microspikes when cells were plated onto laminin (data not shown).

Fig. 4. Rolipram leads to reduced RhoA activity in REF52 cells. (A) REF52 cells were allowed to adhere to laminin coated plates in the absence (–) or presence (+) of rolipram. Cells were lysed 1 hour after plating. The level of active GTP-RhoA was determined by incubating lysates with GST-C21 and analysing the amount of bound RhoA on a western blot. Aliquots of total lysates were also probed with anti-RhoA antibodies to control for total amount of RhoA protein. The level of active RhoA was quantified by densitometric analysis of western blots – the amount of active RhoA was normalised to the amount of total RhoA. Results shown are from cells plated in the absence (black bars) or presence (white bars) of rolipram and of three independent experiments±s.e.m. (B) Cells were left untransfected, transfected with a dominant negative RhoA construct (N19RhoA) or treated with exoenzyme C3 transferase (10 μg/ml in media for 24 hours). Cells were then plated onto laminin under control conditions, fixed 1 hour after plating and stained for actin using TRITC-phalloidin. Arrows indicate actin microspikes. N19RhoA transfectants were identified using an antibody that recognises the N-terminal Myc tag of this protein (not shown). Scale bar, 20 μm. (C) Cells were allowed to adhere to laminin coated chamber slides in the presence of carrier control DMSO (0.1%), LPA (20 μg/ml), rolipram (10 μM) or LPA plus rolipram. Cells were fixed 1 hour after plating and stained for Scar1 (green) and actin (red). Scale bar, 40 μm. The lower panel represents a quantification of cells displaying protrusive microspikes under each condition. Results represent the mean±s.e.m. of three independent experiments.

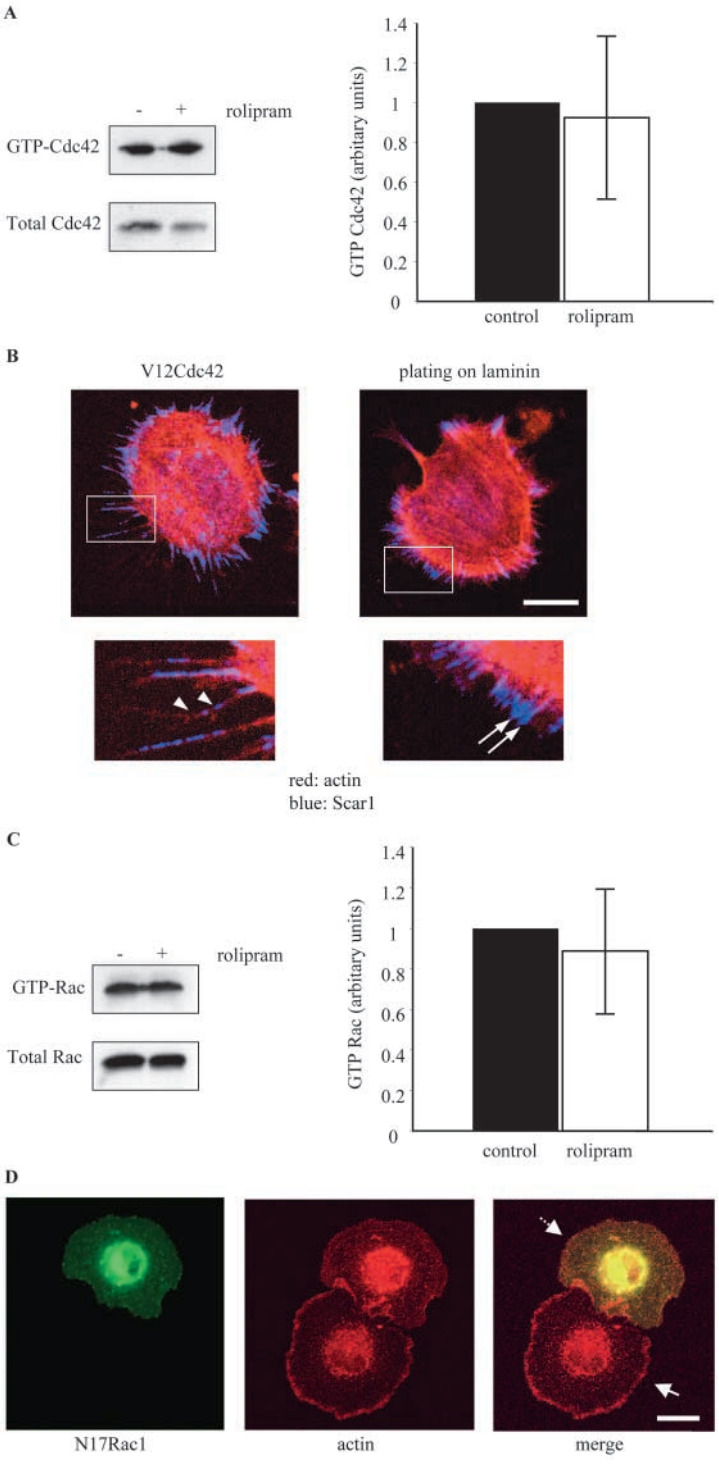


Fig. 5. Rolipram does not alter Cdc42 or Rac1 activity in REF52 cells. (A) REF52 cells were allowed to adhere to laminin coated plates in the absence (–) or presence (+) of rolipram. Cells were lysed 1 hour after plating and the level of active GTP-Cdc42 was determined by incubating lysates with GST-PAK and analysing the amount of bound Cdc42 by western blotting. Aliquots of total lysates were also probed with anti-Cdc42 antibodies to control for total amount of Cdc42 protein. The level of active Cdc42 was quantified by densitometric analysis of western blots – the amount of active Cdc42 was normalised to the amount of total Cdc42. Results of three independent experiments±s.e.m. are shown from cells plated in the absence (black bars) or presence (white bars) of rolipram. (B) Cells transfected with an active mutant of Cdc42 (V12Cdc42) were fixed and stained for Scar1 (blue) and actin (red). V12Cdc42 transfectants were identified with an antibody that recognises the N-terminal Myc-tag of this protein (not shown). In a parallel experiment, non-transfected cells were plated onto laminin, fixed 1 hour after plating and stained for Scar1 (blue) and actin (red). Arrowheads indicate filopodia, which differ structurally from actin microspikes (arrows). Scale bar, 20 μm. (C) Experimental set-up like in (A) except that a Rac1-specific antibody was in the western blot. (D) Cells were transfected with a dominant-negative mutant of Rac1 (N17Rac1). Cells were plated onto laminin in the presence of rolipram. Cells were fixed 1 hour after plating and stained for transfected protein (anti-Myc, green) and actin (red). Solid arrow indicates extensive lamellipodia present in untransfected cell but absent from cell expressing N17Rac1 (dotted arrow). Scale bar, 20 μm.

Expression of well characterised mutant forms of Rac1 that were either dominant inhibitory (N17Rac1) or constitutively active (L61Rac1) had no effect on peripheral microspike assembly (data not shown). However, the extensive lamellipodia around the entire periphery of rolipram-treated cells were Rac1-dependent (Fig. 5D), because over-expression of a dominant-negative Rac1 mutant (N17Rac1) prevented the recruitment of actin and focal complex proteins to these structures (shown here for actin in Fig. 5D, dotted arrow). It is interesting that these structures form in the absence of any increase of levels of GTP-Rac1, indicating that under control conditions, RhoA-induced actin adhesion microspikes are the predominant peripheral actin structures. However, when RhoA is inhibited by blocking PDE4 activity, Rac1-mediated lamellipodia become the default peripheral actin structures.

Signalling downstream of RhoA

The decrease in GTP-RhoA levels in cells treated with rolipram, together with the similar adhesion-phenotypes of rolipram and the inhibition of RhoA activity, are indicative of an important role for RhoA during the assembly of the peripheral actin adhesion structures. RhoA has several downstream effectors, including the Rho kinase ROCK (also known as ROK-α) (Amano et al., 1997; Ishizaki et al., 1997). Indeed, Y27632, a selective inhibitor of ROCK (Uehata et al., 1997) had an effect similar to rolipram on integrin-dependent cell morphology, suggesting that ROCK is a key RhoA effector in this context (Fig. 6A). ROCK phosphorylates and inactivates myosin phosphatase, leading to enhanced phosphorylation of myosin light chain (MLC) at the MLC kinase (MLCK) phosphorylation site, Ser19 (Kimura et al., 1996). In turn, myosin activity, induced by MLC phosphorylation, generates a contractile force that is thought to promote integrin clustering and focal adhesion assembly (Burridge et al., 1997). It was indeed shown that plating REF52 cells onto ECM can increase phosphorylation of MLC at Ser19 (Fincham et al., 2000). Here we noted that, in cells plated onto laminin, rolipram treatment reduced the levels of MLC phosphorylation [judged by immunoblotting (Fig. 6B)] and that this was because localised MLC phosphorylation was suppressed at the cell periphery [judged by immunofluorescence (Fig. 6C,D)]. Specifically,

whereas only a small rolipram-induced reduction in total cellular phospho-MLC was evident (Fig. 6B), there was no visible phospho-MLC staining at the periphery of treated cells (Fig. 6D) when compared to their untreated counterparts (Fig. 6C). Instead, phospho-MLC staining was only visible in cytoplasmic structures of rolipram-treated cells (Fig. 6D, dotted arrows). Some cytoplasmic staining in similar structures was also visible in untreated cells (Fig. 6C, dotted arrows). Y27632 and rolipram had identical effects on phospho-MLC distribution, with staining being detected only in cytoplasmic structures (Fig. 5E, dotted arrows).

To determine how tightly linked rolipram action is to PKA activation and to signalling events downstream of RhoA, such as phospho-MLC recruitment, we carried out dose response analyses for rolipram and Y27632. We found a strong correlation between the suppression of both phospho-MLC localisation to peripheral sites and the formation of actin adhesion microspikes when rolipram concentration was increased; PKA activation occurred in a reciprocal dose-responsive manner (Fig. 7A). In addition, we obtained very similar dose response effects for the ROCK inhibitor on actin adhesion microspike and phospho-MLC suppression (Fig. 7B). These data strengthen the argument that rolipram and the ROCK inhibitor Y27632 are affecting microspike formation in a similar manner by inhibiting peripheral phospho-MLC recruitment downstream of RhoA. A rolipram concentration of 0.05 μM inhibits actin adhesion microspike formation and peripheral phospho-MLC localisation by ~50%. This concentration of rolipram reduces the level of PDE activity in REF52 lysate to 50.4 ± 1.7% in an in vitro PDE assay (data

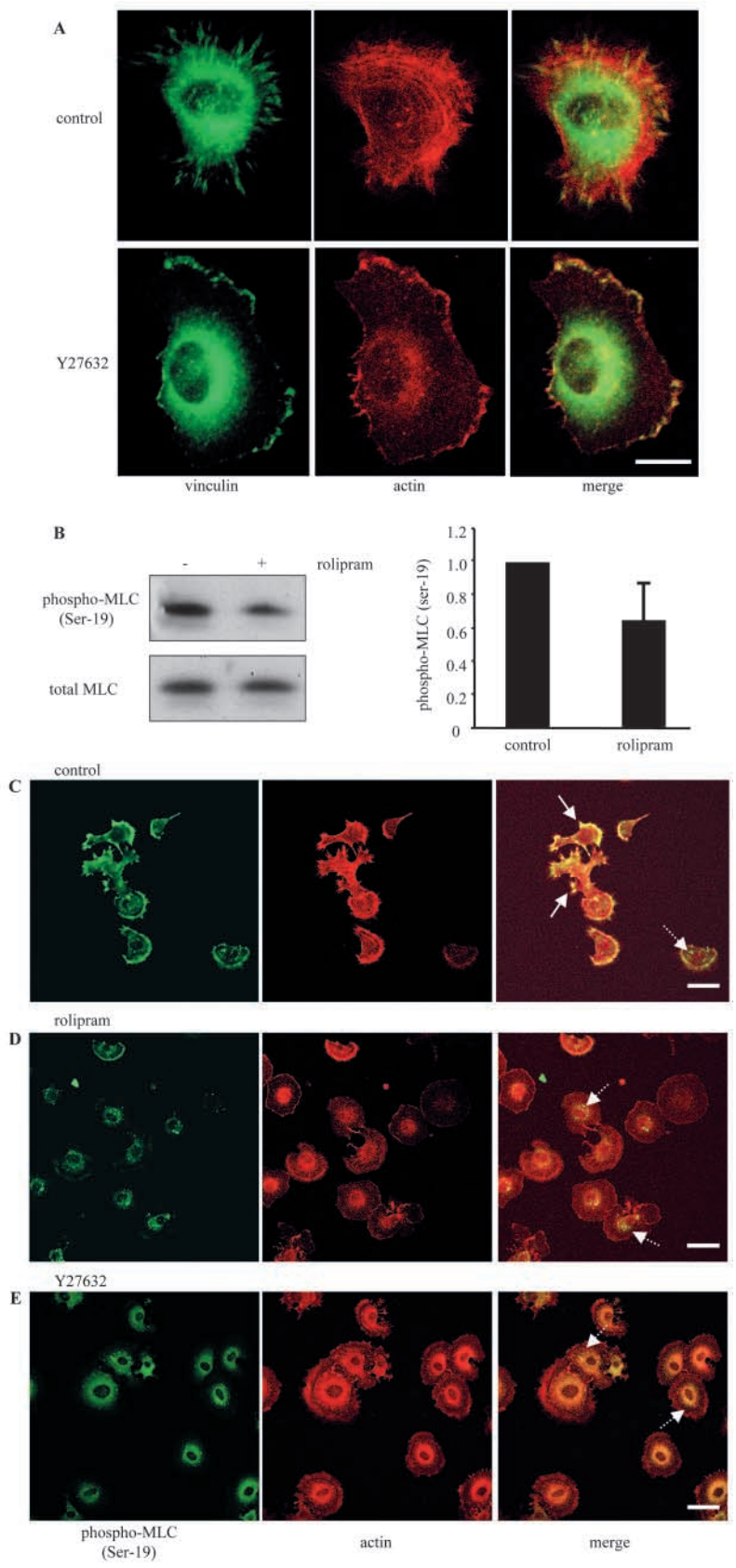


Fig. 6. RhoA effectors ROCK and MLCK mediate assembly of rolipram-sensitive adhesion microspikes. (A) Cells were plated onto laminin under control conditions (upper panel) or in the presence of the ROCK inhibitor Y27632 (10 μM) (lower panel). Cells were fixed 1 hour after plating and stained for vinculin (green) and actin (red). (B) Cells were allowed to adhere to laminin coated plates in the absence (–) or presence (+) of rolipram. Cells were lysed 1 hour after plating and the phosphorylation state of MLC was determined by western blotting using an antibody specific for MLC phosphorylated at Ser19. Levels of total MLC were determined by using an antibody that recognises unphosphorylated and phosphorylated MLC equally well to indicate equal loading. The right panel represents the data of three independent experiments (mean±s.e.m.); phospho-MLC levels were determined by densitometry and normalised to the amount of total MLC. (C) Cells were plated onto laminin under control conditions. After 1 hour cells were fixed and stained for MLC phosphorylated at Ser19 (green) and actin (red). (D) Experimental set-up like in (C) except that rolipram (10 μM) was included. (E) Experimental set-up like in (C) except that Y27632 (10 μM) was included. Solid arrows indicate peripheral phospho-MLC staining only observed in the absence of inhibitors (C). Cytoplasmic phospho-MLC staining, observed under all conditions, is indicated by dotted arrows (C-E). Scale bars, 20 μm (A) and 40 μm (C-E).

not shown; *n*=2). Therefore, a further correlation exists between the inhibition of PDE4 activity, phospho-MLC localisation and actin adhesion microspike formation.

Taken together, our data imply that the inhibition of PDE4 results in impaired peripheral localisation of phospho-MLC, most probably as a consequence of decreased RhoA and ROCK activities. However, it is possible that reduced MLCK activity contributes to this effect because MLCK is also subjected to an inhibitory regulation by PKA, occurring as a consequence of elevated cAMP levels (de Lanerolle et al., 1984; Nishikawa et al., 1984).

Effect of PDE4 inhibition on cell migration

Actin re-modelling, particularly in response to integrin engagement, is a critical component of the cellular contractile machinery that drives cell migration. In particular, RhoA has been shown to have an important role in cell migration in several cell types (Takaishi et al., 1993; Ridley et al., 1999). Our data, using time-lapse video microscopy, show that REF52 cells on ECM components extend and retract microspikes as the cells propel themselves forwards. Representative time-lapse images demonstrate peripheral spikes and evidence of forward migration of REF52 cells (Fig. 8A). In the presence of rolipram, however, protrusive spikes were not visible and the cells had extensive lamellipodia similar to those observed in our ECM plating experiments (Fig. 8B, compare with Fig. 1). Rolipram treatment resulted in change of cell morphology and was accompanied by impaired migration (Fig. 8B); see Movies 1 and 2, http://jcs.biologists.org/supplemental/ for the actual videos showing migration of untreated and rolipram-treated REF52 cells from which these still images were taken. Quantification of random cell movement over the observation period demonstrated that rolipram impaired the rate of random cell migration of REF52 cells (Fig. 8C). These data demonstrate that PDE4 activity is important for re-modelling of the actin cytoskeleton associated with the dynamic regulation of peripheral actin spikes, which, in turn, is necessary for cells to move.

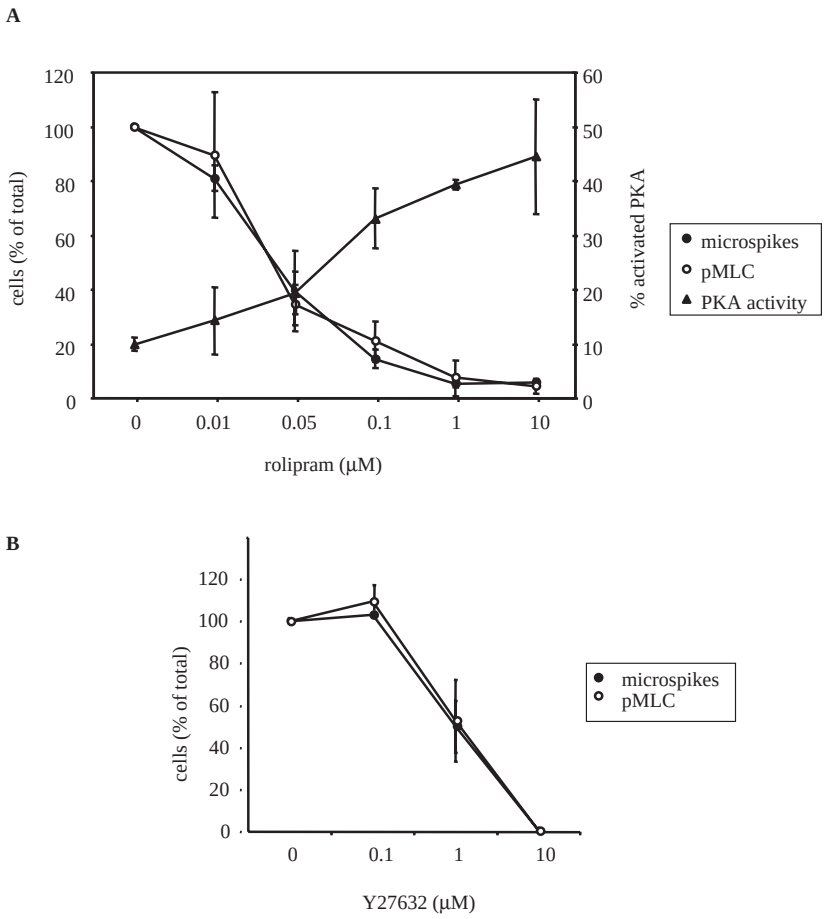


Fig. 7. Loss of actin adhesion microspikes in reponse to rolipram or Y27632 correlates with decreased peripheral phospho-MLC and increased PKA activity. (A) REF52 cells were plated onto laminin in the presence of increasing concentrations of rolipram. Cells were fixed 1 hour after plating and stained for either Scar1 or MLC phosphorylated at Ser19. The number of cells that had formed actin adhesion microspikes under each condition – as judged by Scar1 staining – was determined and expressed as the percentage of total cells plated. Similarly, cells were scored for peripheral staining of phosphorylated MLC under each condition. In parallel experiments, cells were lysed and levels of PKA activity determined using a peptide substrate. The results shown represent the mean±s.e.m. of three independent experiments. (B) Experimental set-up like (A) except that cells were plated in the presence of increasing concentrations of Y27632.

Discussion

How can integrin-induced fluctuations in cAMP alter cellular responses?

Here, we show that control of cAMP degradation can have profound effects on integrin-induced signalling and biological responses. Specifically, inhibition of PDE4 activity suppresses the assembly of dense cortical actin and actin adhesion microspikes in REF52 cells plated on ECM components (Fig. 1). Our results indicate that this occurs through the action of PKA (Figs 2 and 3). Interestingly, there is a growing body of evidence that PKA negatively regulates RhoA function (Laudanna et al., 1997) by direct phosphorylation (Lang et al., 1996; Dong et al., 1998; Tamma et al., 2003; Ellerbroek et al., 2003). PKA-induced phosphorylation of RhoA as a consequence of PDE4 inhibition provides a probable mechanism for the reduced RhoA-GTP levels we observed in REF52 cells (Fig. 4) and the subsequent effects on peripheral microspike formation. It is possible, however, that other PKA-mediated phosphorylation events that we observed, such as phosphorylation of the actin regulatory protein VASP (Fig. 2) also play a role in this process.

RhoA activation in response to cell adhesion is known to be biphasic, with RhoA activity initially decreasing before being reactivated (Ren et al., 1999). Transient depression of RhoA activity is thought to temporally relieve contractile forces and allow cytoskeletal re-arrangements required for integrin complex assembly. Following integrin engagement, intracellular cAMP levels increase, consequently activating PKA (Meyer et al., 2000; O'Connor et al., 2001; Whittard and Akiyama,

2001). Therefore, fluctuations in cAMP might contribute to the transient depression of RhoA activity, perhaps via PKA-mediated phosphorylation of RhoA after integrin engagement. One possibility supported by our results is that a major function of PDE4 activity in these cells is to bring elevated cAMP back to basal levels, resulting in the inactivation of PKA, re-activation of RhoA and the formation of contractile actin adhesion structures that are necessary for the ensuing biological effects.

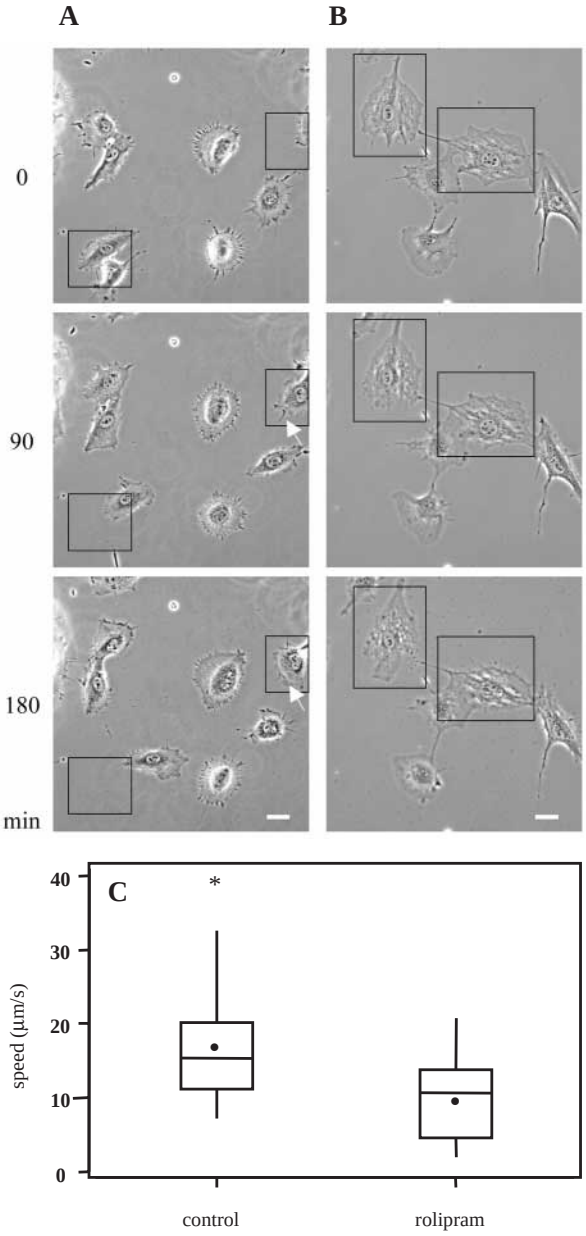


Fig. 8. Rolipram inhibits migration that is associated with dynamic regulation of microspikes. Cells were cultured on laminin coated glass bottomed dishes for 24 hours before being transferred to serum-free media. (A) Carrier (0.1% DMSO) or (B) rolipram (10 μM) was added and cell behaviour observed by timelapse microscopy; images were taken every 5 minutes for 6 hours (see Movies 1, 2, http://jcs.biologists.org/supplemental). (A,B) Stills from those movies, taken at 90-minute intervals. Positions of two cells in each time-series are boxed. Position of a motile peripheral microspike is shown in A (arrows). Scale bar, 40 μm. (C) The range of cell speeds observed under each condition is represented as a box and whisker plot in which the median (solid line across box), mean (•) and outliers (*) are indicated. Statistical analysis of the two data sets (control, n=31; rolipram, n=28) indicated that the reduced rate of random cell movement in the presence of rolipram was highly significant (Student's t-test, P<0.001).

PDE4 inhibition suppresses migration and actin adhesion microspike assembly in REF52 cells

Dynamic re-modelling of the actin cytoskeleton and associated adhesions lead to the assembly of peripheral structures that provide protrusive and contractile forces necessary for cells to move. Actin organisation, in turn, is controlled by the Rho family of small GTPases, particularly Rac1, Cdc42 and RhoA, which induce lammelipodia, filopodia and bundled acto-myosin filaments (stress fibres), respectively. Together with local actin polymerisation, the coordinated spatial and temporal activation and de-activation of the Rho GTPases controls cellular responses to motogenic stimuli in most cell types (reviewed in Mitchison and Cramer, 1996; Nobes and Hall, 1995). Here, we establish, for the first time, that treatment of REF52 cells with the specific PDE4 inhibitor rolipram can suppress cell migration and the formation of peripheral, integrin-dependent, protrusive actin adhesion structures (Figs 1 and 7). In these cells, migration is accompanied by formation and retraction of the actin adhesion spikes that are associated with both PDE4 and RhoA activities, whereas residual Rac1 and membrane ruffling activity after rolipram treatment is not sufficient to drive forward cell locomotion (Figs 1 and 7). Previous studies have shown that in primary rat embryo fibroblasts, Rac1 is essential for the protrusion at the leading edge and forward-cell movement, whereas migration is enhanced by an inhibitor of the RhoA effector kinase ROCK (Nobes and Hall, 1999), suggesting that RhoA activity in these cells actually impairs cell migration. Taken together, the data imply that while RhoA is needed for force generation during migration, it can be inhibitory in some situations, and it is likely that the balance between levels of Rac1 and RhoA activities is important. This is consistent with optimal migration requiring intermediate levels of RhoA-induced force-generation and adhesiveness (reviewed in Lauffenburger and Horwitz, 1996); in the case of REF52 cells, integrin-dependent, PDE4- and RhoA-dependent peripheral actin/adhesion structures are associated with forward cell migration.

Allergic airway diseases are associated with cells of the immune system migrating into the lung. For example, asthma is associated with the infiltration of eosinophils, whereas in chronic obstructive pulmonary disease (COPD) neutrophils are the major infiltrating cell type. PDE4 inhibitors can attenuate neutrophil/eosinophil migration into the lung in models of these diseases (Spina et al., 1998; Torphy et al., 1998; Giembycz, 2000). In particular, it has been demonstrated that rolipram inhibits eosinophil chemotaxis in vitro (Alves et al., 1996; Tenor et al., 1996) and trafficking to sites of inflammation in vivo (Cooper et al., 1999). Rolipram also suppresses many aspects of neutrophil function including adhesion to endothelial cells (Derian et al., 1995). The results described here establish a novel role for PDE4s in the

regulation of the actin cytoskeleton and, as a consequence, cell migration. These observations might contribute towards the understanding of PDE4 activity in immune cell migration during allergic airway diseases (Spina et al., 1998; Torphy et al., 1998; Giembycz, 2000). The results described here might also have impact on cancer therapy; deregulation of cell migration is pivotal to the invasive and metastatic properties of cancer cells, manipulation of PDE4 activity might provide a novel therapeutic cancer target.

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