Protein kinase C-δ C2-like domain is a binding site for actin and enables actin redistribution in neutrophils

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Neutrophils play a key role in host-defence mechanisms against invading pathogens, using their capacity to migrate, engulf micro-organisms and produce toxic radicals. Protein kinase C (PKC) isotypes are important intracellular regulators of these processes in neutrophils. PKC isotypes themselves are controlled by interactions with lipids, Ca^{2+} and proteins. The C2-like domain of PKC-δ (δC2) has been identified as a protein-interaction domain in this PKC isotype. In the present paper we have investigated the contribution of protein interactions at this domain to the regulation/function of PKC-δ in neutrophils. Using affinity chromatography we identified actin as a δC2 binding partner in these cells. Fluorescein-labelled δC2, microinjected into immobilized neutrophils, interacts with filamentous actin (F-actin) inside the cell. PKC-δ co-localizes with F-actin in neutrophils, in lamellipodia at the leading edge of the cell. Stimulation with phorbol ester or IgG-opsonized *Staphylococcus aureus* results in co-ordinated redistribution of PKC-δ and F-actin, and a PKC-δ inhibitor inhibits these changes. Microinjection of δC2 also inhibits F-actin redistribution. Thus PKC-δ binds to F-actin through its C2 domain, and these interactions are important in regulating actin redistribution in neutrophils.

Key words: cytoskeleton, microinjection, migration, protein interaction, rottlerin.

INTRODUCTION

Polymorphonuclear leucocytes (neutrophils) form a major component of the white-blood-cell mass in the body. They play a key role in innate defence mechanisms against invading pathogens, using their capacity to migrate, engulf micro-organisms and produce toxic radicals. Receptors on the cell surface regulate these processes by activating a cascade of intracellular reactions. An important role in this signal transduction process is played by protein kinases, which phosphorylate their downstream targets to transduce receptor information. The serine/threonine kinase protein kinase C (PKC) is a main target for the second messengers diacylglycerol and Ca^{2+}, which are generated upon receptor activation [1–5]. The potential relevance of PKC for neutrophil function has been shown using phorbol esters, which bypass the receptor and mimic the action of diacylglycerol to activate PKC directly. Phorbol esters stimulate the formation of toxic radicals by the NADPH oxidase, degranulation, cell adherence, migration, actin assembly and the activation of ionic currents [6–10].

'PKC' represents a 'family' of enzymes (termed isotypes) rather than a single enzyme. Three PKC subfamilies can be distinguished based on topology: classical PKCs (PKC-α, -$\beta_{\rm I}$, -$\beta_{\rm II}$ and -γ), novel PKCs (PKC-δ, -ε, -η and -θ) and atypical PKCs (PKC-ζ and -ι/λ) [1]. This subdivision is based on the presence of conserved domains acting as binding sites for diacylglycerol (C1 domain; also binds phorbol ester [11–15]) and Ca^{2+} (C2 domain [16–18]). Classical PKCs are regulated by both messengers since they contain a C1 domain and a C2 domain. Novel PKCs have a C1 domain; however, they lack a Ca^{2+}-binding C2 domain. By crystallographic analysis we confirmed that a C2-like domain is present in PKC-δ (δC2). It has the overall features of a C2 domain but lacks essential Ca^{2+}-co-ordinating loops [19]. This indicates that, at least in PKC-δ, the C2 domain has a function different from binding Ca^{2+}. Atypical PKCs contain neither a C1 domain nor a C2 domain, and are not sensitive to Ca^{2+} or diacylglycerol.

Our own observations indicate that, *in vitro*, δC2 is a site of protein interaction [20], mediating the binding of the substrate GAP-43 ('growth-associated protein of 43 kDa'). Mochly-Rosen and co-workers ([21,22], but see [22a], [23,24]) showed that the C2 counterparts in PKC-ε and PKC-β are also sites of protein interaction. Thus the combination of protein interactions at δC2 and lipid interactions at δC1 may determine the regulation of PKC-δ and the execution of its function.

We and others have shown that neutrophils contain at least PKC-β and PKC-δ [6,25–27]. Evidence to date suggests that the PKC-β isotype is important for regulation of the NADPH oxidase, whereas PKC-δ may have other functions [6,28]. The purpose of the present study was to identify protein partners interacting with δC2 so as to derive information on the cellular function of PKC-δ. We used affinity chromatography and established that actin is the major intracellular δC2-binding protein in neutrophils. Endogenous PKC-δ in these cells is associated with the cellular filamentous actin (F-actin) pool, in lamellipodia at the leading edge of the cell. We recently developed a technique that enables microinjection of these primary cells, and show in the present paper that microinjection of δC2 results

Abbreviations used: δC2, C2-like domain of PKC-δ; F-actin, filamentous actin; G-actin, globular actin; his-δC2, histidine-tagged δC2; MALDI–TOF, matrix-assisted laser-desorption ionization–time-of-flight; Ni-NTA, Ni^{2+}-nitrilotriacetate; PKC, protein kinase C; TLCK, tosyl-lysylchloromethane; TRITC, tetramethylrhodamine β-isothiocyanate.

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in disruption of actin remodelling. This data indicates that PKC-δ participates in the regulation of the actin cytoskeleton in neutrophils.

MATERIALS AND METHODS

Materials

Antibodies used in the present study were rabbit anti-(PKC-δ) (C-20 clone; Santa Cruz Biotechnology), tetramethylrhodamine β-isothiocyanate (TRITC)-labelled goat anti-rabbit (Pierce) and FITC-labelled goat anti-rat (Sigma). Other compounds used were Probond Ni^{2+}-nitrilotriacetate (Ni-NTA) agarose beads (Qiagen), Superdex™ 75 prep grade resin, HiTrap™, HiLoad™ 16/60 and Superdex 200 columns (Pharmacia), Hepes-buffered Ham's F-12 culture medium, TRITC-labelled phalloidin and rabbit muscle actin (Sigma), non-muscle actin (85% β-actin/15% γ-actin) (Cytoskeleton, Denver, CO. U.S.A.) and Fluor-X™ monofunctional reactive dye (Amersham).

6 × Histidine-tagged δC2 (his-δC2) purification

BL-21 (DE3) *Escherichia coli* cells were cotransfected with pET14b-δC2 [29] and a pREP4 plasmid coding for the LacI repressor. Cells were grown overnight in 100 ml of Luria–Bertani medium, transferred to 4 litres of expression medium [3.5 g/l KH_2PO_4, 5.0 g/l K_2HPO_4, 3.5 g/l $(NH_4)_2PO_4$, 10 g/l yeast extract, 0.5 g/l $MgSO_4$ and 10 g/l glucose plus 50 mg/l kanamycin and 100 mg/l ampicillin] in a fermentor (Electrolab, Tewkesbury, Gloucestershire, U.K.), grown to an attenuance (D_{600}) of 1.5 before induction with 500 μM isopropyl β-D-thiogalactoside, and were allowed to continue growing until they reached an attenuance (D_{600}) of 12–15. Bacteria were then harvested and lysed by sonication in buffer A [50 mM Tris/HCl (pH 7.9), 300 mM NaCl, 10% (v/v) glycerol, 5 mM 2-mercaptoethanol and 0.25 mM PMSF]. Bacterial debris was removed by centrifugation, and the supernatant, containing soluble his-δC2 was adsorbed on to Ni-NTA agarose beads for 1 h at 4 °C. After two washes with buffer A and four washes with 50 mM Tris/HCl (pH 7.9), 300 mM NaCl, 10 mM imidazole, 20% (v/v) glycerol and 5 mM 2-mercaptoethanol, his-δC2 was eluted with 1 ml of 50 mM Tris/HCl (pH 7.9), 100 mM NaCl, 20% (v/v) glycerol, 500 mM imidazole, 5 mM 2-mercaptoethanol and 10 mM benzamidine. Minor contaminating proteins were removed by gel filtration using a HiLoad™ 16/60 column with a Superdex™ 75 prep grade gel in buffer B [50 mM Tris/HCl (pH 7.9), 300 mM NaCl and 10 mM benzamidine].

To remove the histidine tag, his-δC2 was cleaved with 10 units of thrombin/100 μg of his-δC2 in 20 mM Tris/HCl (pH 8.4), 150 mM NaCl and 2.5 mM $CaCl_2$ for 2 h at 37 °C. Digested protein was purified by gel filtration as described above and the purity was confirmed by SDS/PAGE and Coomassie Brilliant Blue staining of the gel. A single band was observed on the gel for all preparations used in the experiments. For microinjection experiments, the isolated δC2 was labelled with the green fluorescent dye Fluor-X under the conditions specified by the manufacturer and was purified by gel filtration as described above.

Neutrophil isolation

Neutrophils were isolated from buffy coat blood or fresh blood donated by healthy donors by dextran sedimentation and single-step separation over a Ficoll-hypaque solution [30]. Contaminating erythrocytes were lysed by hypotonic shock in water for 15 s and removed by centrifugation. Neutrophils were washed with 0.15 M NaCl and buffer C (0.15 M NaCl, 10 mM $NaHPO_4$ and 10 mM KCl, pH 7.3), and were resuspended in RPMI 1640 or Hepes-buffered Ham's F-12 culture medium.

Affinity experiments

Neutrophils were incubated at 37 °C in RPMI 1640 medium and stimulated for 2 min with IgG-opsonized *S. aureus*. The reaction was stopped by adding 10 ml of ice-cold buffer C, and the cells were collected by centrifugation at 2000 ***g*** for 20 min at 4 °C. The pellet was resuspended in buffer D [10 mM Pipes (pH 7.4), 100 mM KCl, 5 mM NaCl and 3.5 mM $MgCl_2$] plus phosphatase and protease inhibitors [10 mM benzamidine, 50 mM NaF, 10 mM β-glycerophosphate, 2 μg/ml aprotinin, 1 μM pepstatin A, 100 μM tosyl-lysylchloromethane (TLCK) and 100 μM leupeptin]. Cells were homogenized in a Dounce homogenizer by 25 up-and-down strokes, followed by sonication (three 10 s bursts) at 4 °C and incubation for 30 min at 4 °C. Nuclei and cell debris were removed by centrifugation at 1000 ***g***. The supernatant was centrifuged at 100000 ***g*** for 30 min, obtaining a cytosolic fraction (S-100) and a microsomal fraction (pellet). To obtain the S-500 fraction, the S-100 fraction was further centrifuged at 500000 ***g*** in a TLX ultracentrifuge (Beckman).

The S-100 or S-500 fraction was preincubated with 0.2 vol. of buffer B with or without non-tagged δC2 for 1 h at 4 °C, followed by incubation with his-δC2-loaded Ni-NTA for 1 h at 4 °C. The beads were collected by centrifugation and washed twice with buffer D containing 10 mM benzamidine and four times with buffer D containing 10 mM benzamidine and 10 mM imidazole. Bound proteins were eluted with buffer D containing 10 mM benzamidine and 500 mM imidazole. The eluate was mixed with buffer E [62.5 mM Tris/HCl (pH 6.8), 2% (w/v) SDS, 2.5% (v/v) glycerol, 1 mM 2-mercaptoethanol and 0.025% (w/v) Bromophenol Blue] and boiled for 5 min. Proteins were separated by SDS/PAGE [15% (w/v) polyacrylamide] and stained with Coomassie Brilliant Blue.

In vitro binding experiments

Muscle actin or non-muscle actin at 0.4 mg/ml was maintained in globular actin (G-actin) buffer [5 mM Tris/HCl (pH 8.0), 2 mM $CaCl_2$, 2 mM ATP and 0.5 mM dithiothreitol]. In F-actin-binding experiments, 10 × F-actin buffer was added (where 1 × F-actin buffer corresponds to 50 mM KCl, 2 mM $MgCl_2$ and 1 mM ATP) and this mixture was incubated for 1 h at 20 °C to polymerize. F- or G-actin, his-δC2 and δC2 at concentrations indicated in the text and Figure legends were mixed in a volume of 100 μl and incubated for 1 h at 37 °C. Ni-NTA beads (bed volume of 5 μl) were added and tubes were rotated for 30 min at 37 °C. Beads were then recovered by centrifugation, the supernatant was removed and the beads were washed twice with F- or G-actin buffer and twice with these buffers containing 10 mM imidazole. Elution was performed with F- or G-actin buffer containing 500 mM imidazole for 30 min. The eluate was mixed with buffer E, boiled for 5 min and proteins were resolved by SDS/PAGE and visualized by silver staining.

Tritron extractions

For Triton extractions, intact neutrophils were homogenized in 20 mM Tris/HCl (pH 7.4), 1% (v/v) Triton X-100, 2 μg/ml aprotinin, 100 μM TLCK, 1 μM pepstatin, 50 μg/ml PMSF and

1 μg/ml di-isopropylfluorophosphate by 20 up-and-down strokes in a Dounce homogenizer. The extract was incubated at 4 °C for 15 min and centrifuged at 22000 ***g*** at 4 °C. The supernatant was taken (Triton-soluble) and the pellet was re-extracted as above. After clearance, the supernatant was discarded and the pellet was homogenized and sonicated in buffer E (Triton-insoluble). To the Triton-soluble fractions 0.2 vol. of five times concentrated buffer E was added. Each fraction (10 μl) was analysed by SDS/PAGE and Western blotting for the presence of PKC-δ as described previously [20].

Matrix-assisted laser-desorption ionization–time-of-flight (MALDI–TOF) MS

Coomassie Brilliant Blue-stained proteins were taken from the gel, destained and digested as previously described [31]. Proteins were destained by incubation with 40 % ethanol plus 50 mM NH_4HCO_3. Once destained, protein bands were cut into small pieces and soaked twice in 25 mM NH_4HCO_3 for 15 min at 30 °C. The fluid was removed and gel pieces were incubated three times for 10 min with acetonitrile. Gel pieces were completely dried under vacuum, after which they were re-hydrated by addition of 150–300 ng of trypsin (Boehringer Mannheim) in 25 mM NH_4HCO_3. Finally, 10–15 μl of 25 mM NH_4HCO_3 was added to cover the gel pieces and the mixture was incubated overnight at 30 °C. For MALDI–TOF MS, 0.5 μl of the total digestion solution was applied to the target disk and allowed to air dry. This was overlaid with 0.5 μl of matrix solution [1 % (w/v) α-cyano-4-hydroxycinnamic acid in 50 % (v/v) acetonitrile/0.1 % (v/v) trifluoroacetic acid] and left to dry again. Peptide masses were obtained by using a Biflex III MALDI–TOF mass spectrometer (Bruker). For interpretation of the MALDI spectra, a list of mass-to-charge ratios was acquired for each protein digest and processed using four different browsers (MS-Fit at falcon.ludwig.ucl.ac.uk/ucsfhtml3.2/msfit.htm, Peptident at www.expasy.ch/tools/peptident.html, Mascot at www.matrixscience.com and ProFound at prowl.rockefeller.edu/cgi-bin/ProFound) and analysed by comparison against three separate databases [Owl, National Center for Biotechnology Information ('NCBI') and SwissProt]. Mass tolerance was limited to a relative value of 200 p.p.m. and proteins were matched according to probability scores deduced by each browser.

Microinjection

Neutrophils were resuspended in Hepes-buffered Ham's F-12 culture medium, spotted on fibronectin/collagen-coated coverslips and allowed to set for at least 30 min at 37 °C. Fluor-X-labelled δC2 (50–200 ng/ml) was diluted in buffer F [1 mM Hepes (pH 7.4), 100 mM KCl and 5 mM $MgCl_2$], loaded in the microinjection syringe (Clark Electromedical Instruments, Pangbourne, Reading, U.K.) and injected into cells using an Eppendorf transjector 5246 and an Eppendorf micromanipulator 5171. During microinjection, cells were continuously maintained at 37 °C, under an atmosphere of 10 % CO_2. Following microinjection, cells were kept for 20 min at 37 °C and activated with PMA for 5 min. After activation, cells were washed with buffer G (buffer C supplemented with Ca^{2+} and Mg^{2+}) and fixed with 4 % (v/v) formaldehyde in buffer G for 30 min.

Immunofluorescence

Microinjected cells were fixed as indicated earlier and were washed six times in buffer G and permeabilized for 5 min with 0.2 % Triton X-100 in buffer G. After four washes with buffer G, cells were immersed in 0.1 μg/ml TRITC-labelled phalloidin. Coverslips were cultured in the dark and incubated for 45 min. After two new washes with buffer G, coverslips were mounted with 5 μl Mowiol (Calbiochem) plus 0.1 % *p*-phenylenediamine to prevent photobleaching.

Non-microinjected cells were stained with antibodies as described previously [6], with minor modifications. Cells were plated on glass or fibronectin/collagen-coated coverslips for 1 h. In some cases, cells were preincubated with 10 μM rottlerin for 30 min at 37 °C. Cells were subsequently incubated with the indicated concentrations of PMA, washed and fixed in buffer G containing 4 % (w/v) paraformaldehyde and 0.1 % glutaraldehyde for 20 min. After four washes with buffer G, cells were permeabilized for 15 min with buffer G containing 0.5 % Triton X-100, washed four times with buffer G and blocked two times for 15 min with buffer G containing 1 mg/ml sodium borohydride and for 2 h at 22 °C with buffer G containing 5 % (v/v) goat serum. Cells were incubated for 48 h with primary antibody (1:50 dilution) at 4 °C and for 1 h at 20 °C in buffer G containing 5 % (v/v) goat serum. After six washes, incubation with specific FITC- or TRITC-labelled secondary antibodies (1:200 dilution) was performed for 1 h at 20 °C in buffer G containing 5 % (v/v) goat serum, followed by four washes with buffer G, and in the case of F-actin labelling incubation for 40 min at 20 °C with TRITC-phalloidin (1:500 dilution). After four final washes with buffer G, coverslips were mounted in Mowiol [6] plus 0.1 % *p*-phenylenediamine and visualized in a Leica DMRBE confocal microscope using the Leica TCS NT software (version 1.6.587).

RESULTS

δC2 binding partners in neutrophils

In order to identify potential δC2 binding partners in neutrophils, we prepared a solid matrix consisting of his-δC2 immobilized on Ni-NTA agarose beads. These were incubated with cytosols obtained from neutrophils after centrifugation at 500000 ***g*** (S-500 fraction). As a control for non-specific binding, cytosols were preincubated in the presence of an excess of non-tagged, free δC2 and then incubated with the his-δC2-loaded beads. A protein with a molecular mass of 43000 Da was retained on the beads in the absence, but not in the presence, of free δC2, suggesting it interacted specifically with δC2 (Figure 1). This S-500 δC2 binding partner was identified by MALDI–TOF MS as non-muscle actin (Table 1). The MALDI spectrum revealed a tryptic peptide with the same mass/charge ratio as that predicted for β-actin acetylated at the N-terminus. This indicates that the non-muscle actin isoform interacting with δC2 is β-actin and not γ-actin, for which no such peptide was present in the spectra. Performing the same binding experiment using an S-500 fraction from activated neutrophils, resulted in increased specific binding of β-actin to the his-δC2 matrix (Figure 1). When low speed cytosols (S-100) were used for affinity chromatography, a complex of proteins was retained on the δC2 column, including actin and the actin-related protein 2/3 (results not shown).

To determine whether β-actin interacts directly with δC2 we employed purified non-muscle actin (85 % β-actin/15 % γ-actin) in an immobilization binding assay similar to the one used above. G- or F-actin forms of non-muscle actin were incubated together with his-δC2, which, after extensive washing, was eluted from the beads using imidazole. Figure 2(A) shows that both forms of actin bound to δC2. We compared the specific binding of δC2 to non-muscle actin with that to muscle actin (largely the α-actin isoform), since evidence from the literature suggested that PKC

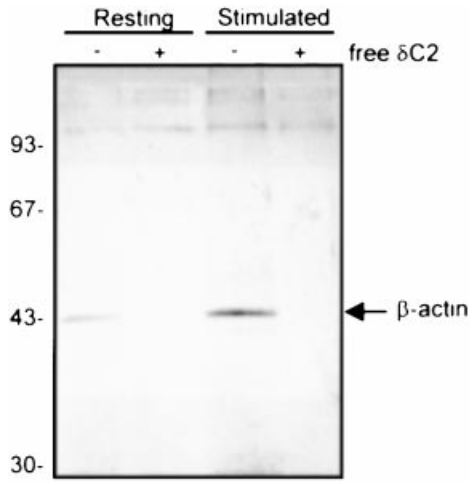

Figure 1 his-δC2 affinity chromatography of the cytosolic S-500 fraction from neutrophils

Neutrophils were incubated for 2 min with vehicle or IgG-opsonized *S. aureus*, recovered, lysed and an S-500 fraction was prepared as described in the Materials and methods section. The S-500 fraction was preincubated in the presence (+) or absence (−) of non-tagged δC2 for 1 h at 4 °C. Extracts were then incubated with his-δC2 and immobilized on Ni-NTA beads for 1 h at 4 °C. After extensive washing, complexes were eluted with 500 mM imidazole and analysed by SDS/PAGE [15% (w/v) polyacrylamide] and silver staining. The positions of molecular mass markers (in kDa) are indicated on the left-hand side.

Table 1 Tryptic peptides observed in the MALDI–TOF spectrum of the excised 43 kDa protein

Observed mass spectra were searched against databases of predicted peptide profiles. The peptide profile expected for β-actin is identical to that observed in the spectrum. For each matching peptide, the position and sequence is shown.

$[M+H]^+$				
Observed	Expected	ΔMass	Position	Peptide
795.55	794.46	0.08	329–335	IIAPPER
800.64	799.53	0.11	62–68	RGILTLK
923.59	923.56	0.02	329–335	IIAPPERK
976.43	975.44	−0.01	19–28	AGFAGDDAPR
998.42	997.48	−0.07	184–191	DLTDYLMK
1036.59	1035.64	−0.06	327–335	IKIIAPPER
1132.48	1131.52	−0.04	197–206	GYSFTTTAER
1161.53	1160.61	−0.09	316–326	EITALAPSTMK
1171.52	1170.56	−0.06	40–50	HQGVMVGMGQK
1187.50	1186.56	−0.06	40–50	HQGVMVGMGQK*
1198.83	1197.70	−0.03	29–39	AVFPSIVGRPR
1203.50	1202.55	−0.06	40–50	HQGVMVGMGQK†
1354.62	1353.62	0.00	51–62	DSYVGDEAQSKR
1515.76	1514.74	0.01	85–95	IWHHTFYNELR
1516.72	1515.70	0.02	360–372	QEYDESGPSIVHR
1644.74	1643.79	−0.06	360–373	QEYDESGPSIVHRK
1790.95	1789.88	0.06	239–254	SYELPDGQVITIGNER
1835.84	1834.81	0.02	2–18	DDDIAALVVDNGSGMCK‡
1851.97	1850.80	0.16	2–18	DDDIAALVVDNGSGMCK§
1954.09	1953.06	0.03	96–113	VAPEEHPVLLTEAPLNPK
2215.26	2214.02	0.19	292–312	DLYANTVLSGGTTMYPGIADR
2231.13	2230.06	0.06	292–312	DLYANTVLSGGTTMYPGIADR*
3183.85	3182.61	0.23	148–177	TTGIVMDSGDGVTHTVPIYEGYALPHAILR

* One methionine residue oxidized.
† Two methionine residues oxidized.
‡ Protein acetylation.
§ Protein acetylation and one methionine residue oxidized.

isotypes interact with the latter actin species [32]. Of the two actin isoforms non-muscle actin interacted with δC2, the apparent interaction of muscle actin being due to non-specific binding to the beads (Figure 2B). This was confirmed in a competition assay as described above. Non-muscle actin binding was competed off the his-δC2-loaded beads by excess δC2, whereas muscle actin failed to be competed. At 1 μM his-δC2 and 1 μM non-muscle actin, the amount of δC2 required to provoke competition to half maximal levels of binding was approx. 0.5 μM (Figure 2C). No such competition occurred for muscle actin (Figure 2D). At higher concentrations of F-actin, binding of muscle actin to δC2 was apparent; however, the level of binding was always lower than that observed for non-muscle actin (Figure 2E). This could be due to a preference of δC2 for interaction with non-muscle actin or to differences in average filament size for the two F-actin species.

To investigate if the interaction between δC2 and actin occurs in the cell, we microinjected Fluor-X-labelled δC2 into neutrophils and determined its colocalization with the actin cytoskeleton by confocal microscopy. As a control, rat IgG was microinjected and further visualized with fluorescein-labelled anti-rat IgG. Microinjected neutrophils showed a characteristic cortical F-actin distribution with a light network of F-actin inside the cytoplasm (Figure 3). Rat IgG showed a diffuse, probably cytoplasmic, localization, since it counter-stained nuclear and intracellular vesicular structures. No relevant colocalization was observed between cortical F-actin and the microinjected rat IgG. By contrast, Fluor-X-labelled δC2 was located in the periphery of the cell and colocalized with cortical F-actin, although a diffuse cytoplasmic staining was also observed (Figure 3).

Taken together, these results demonstrate that β-actin is a binding partner for δC2 with which it interacts directly *in vitro*. Although the colocalization of δC2 and cortical actin may not involve the direct interaction between the two, the *in vitro* binding suggests that this would be the case. Thus δC2 may mediate the interaction of PKC-δ with the actin cytoskeleton in neutrophils.

Colocalization of PKC-δ with F-actin in neutrophils

In order to assess whether δC2 interactions could participate in the interaction of PKC-δ with the actin cytoskeleton in intact neutrophils, we performed cell fractionation and confocal immunofluorescence microscopy experiments. Extraction of neutrophils showed that the amount of PKC-δ in the Triton X-100-insoluble fraction, which contains the stable F-actin pool [33,34], was 51 ± 11 % (mean ± S.D., $n = 4$) of the total amount of PKC-δ in these cells.

Figure 4(A) shows a representative experiment in which the cellular localization of PKC-δ and actin was determined by confocal immunofluorescence microscopy. We used two substrates in these studies, glass and a mixture of fibronectin and collagen. PKC-δ staining was observed in most, but not all, cells. Dependent on the morphology of the cell, PKC-δ localization appeared either diffuse or highly polarized (Figure 4A). Examples of cells in which PKC-δ was polarized are shown in Figure 4(B). A flattened, leading area of the cell could be observed, which contains a significant proportion of the total cellular amount of PKC-δ. Here, PKC-δ appeared diffuse, counter-staining both the nucleus and granular structures. In addition, PKC-δ was present in lamellipodia-like structures at the periphery of the flattened area. These structures also contained F-actin. F-actin staining was observed at the other side of the cell, the trailing area, in bundles and in focal structures. PKC-δ was not apparent in this

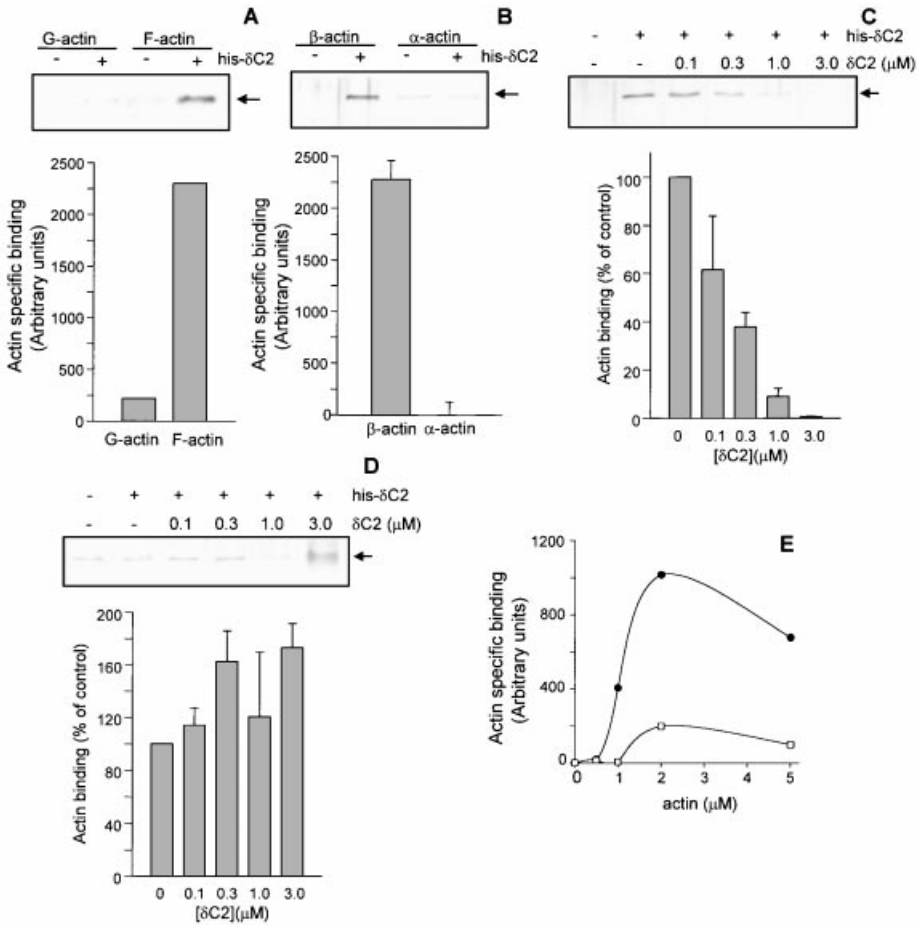

Figure 2 ***In vitro*** **binding of pure non-muscle actin to δC2**

(**A**) Monomers or filaments of β-actin at a concentration of 1 μM were incubated in their respective buffers with 1 μM his-δC2, and specific binding was determined after elution of Ni-NTA beads with imidazole after extensive washing. Specific binding of G- or F-actin (defined as the binding in the presence of δC2 on the beads minus the binding in the absence of δC2 on the beads) to his-δC2 was quantified by densitometry. The result shown is representative of three independent experiments. (**B**) Filaments of β-actin and α-actin were incubated with his-δC2 in F-actin buffer and recovered by elution after binding to Ni-NTA beads. The average specific actin binding $\pm$ S.E.M. ($n = 3$) is shown. (**C**) Inhibition of the binding of 1 μM β-actin filaments to 1 μM his-δC2 by increasing concentrations of non-tagged δC2. The means $\pm$ S.E.M. from three different experiments are shown. (**D**) As (**C**), except that muscle-actin filaments were tested in the assay. (**E**) Concentration dependence of β-actin (●) or α-actin binding (□) to his-δC2. Actin was incubated with 1 μM his-δC2. The complex was bound to Ni-NTA beads and eluted by imidazole. Data represent the means from three different experiments.

area of the cell. The localization and polarization of PKC-δ did not significantly differ between the two substrates used in the present study (Figure 4A).

These localization studies are consistent with our fractionation results, suggesting that PKC-δ is at least in part associated with F-actin. In order to further substantiate the association of PKC-δ with F-actin, we treated the cells with jasplakinolide, an oligopeptide that interrupts the actin cycle and results in accumulation of F-actin. Jasplakinolide treatment resulted in the accumulation of cortical F-actin and concomitantly in the association of almost all cellular PKC-δ with this cortical F-actin component (Figure 4C). Thus the combination of our cell fractionation and immunofluorescence data suggests that PKC-δ in neutrophils associates with F-actin, in particular with the F-actin component that is localized in lamellipodia.

Treatment of neutrophils with PMA did not change the amount of PKC-δ associated with the Triton-insoluble fraction of neutrophils. After treatment with 0.1 μg/ml PMA for 2 min 45 ± 16 % (mean $\pm$ S.D., $n = 4$) of the total PKC-δ content of the cell was present in the Triton-insoluble fraction. However, immunofluorescence analysis showed that parallel dynamic changes occurred in PKC-δ and F-actin. Figure 4(B) shows that, upon PMA treatment, the cells lost the polarized localization of PKC-δ. Instead, PKC-δ-containing, lamellipodia-like structures extended in all directions. As in control cells, PKC-δ and F-actin colocalized in these structures. The structures could be seen readily in the horizontal dimension, surrounding the 'centre' of the cell. However, analysis of the upper layers of the cell by confocal sectioning showed that they also extended in the vertical dimension (Figure 4B), appearing as ruffles on the top of the cell.

Application of IgG-coated *S. aureus* particles to the cells resulted in a dramatic repolarization of PKC-δ and F-actin staining. The flattened area of the cells was no longer visible, but instead all PKC-δ localized towards the area of the cell where particle intake occurs. As shown in Figure 4(B), extensive ruffling occurred in this area, and confocal sectioning revealed co-localization of PKC-δ and F-actin in these ruffles.

Altogether, these results indicate that PKC-δ colocalizes with F-actin in neutrophils, in particular in those areas of the cell where a high degree of F-actin turnover may be expected to take

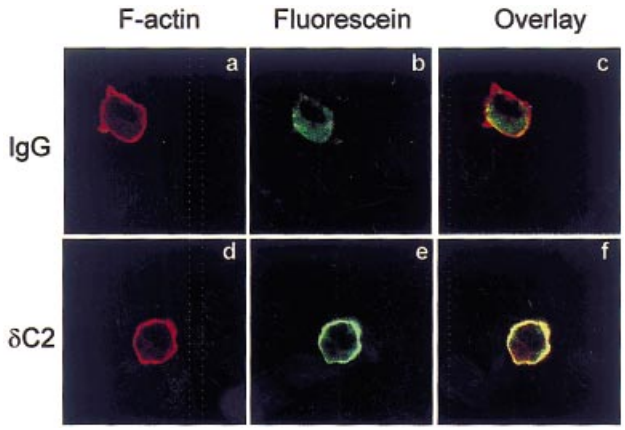

Figure 3 δC2 binds to the actin cytoskeleton in microinjected neutrophils

Neutrophils were seeded on fibronectin/collagen-coated coverslips for 30 min at 37 °C. Cells were then microinjected with rat IgG (**a**–**c**) or Fluor-X-labelled δC2 (**d**–**f**). Microinjected rat IgG was visualized by staining with an FITC-conjugated anti-rat antibody (**a**), whereas δC2 was labelled with FITC before microinjection (**d**). F-actin was visualized using TRITC-phalloidin (**b** and **e**). (**c** and **f**) Show co-localization (in yellow) by overlaying the signals from (**a** and **b**) and (**d** and **e**) respectively.

place, i.e. in the flattened leading cell end of control (migrating) cells, in the peripheral lamellipodia and apical ruffles on PMA-stimulated cells and in the area of particle intake in *S. aureus*-treated cells.

Regulation of neutrophil F-actin polarization by PKC-δ through δC2 interactions

Binding of δC2 to F-actin and the colocalization of PKC-δ with the active pool of cellular actin suggest that PKC-δ may play a role in the dynamics of the actin cytoskeleton in neutrophils. To investigate this further we performed interference experiments.

First we employed rottlerin, an inhibitor that has a preference for inhibiting PKC-δ [35]. Figure 4(B) shows the effect of rottlerin on neutrophils placed on glass coverslips. Treatment of these cells with rottlerin resulted in loss of cell morphology and substantial loss of the phalloidin staining. Rottlerin-treated cells were invariably rounded, and colocalization of PKC-δ with F-actin was no longer apparent. No lamellipodia-like structures were observed. Stimulation of the cells with IgG-coated *S. aureus* did not result in the repolarization of F-actin, suggesting that PKC-δ plays a major role in this cell behaviour (Figure 4B). However, application of PMA to rottlerin-treated cells changed the cell morphology and a substantial flattening of the cells occurred. The appearance of these cells was different from cells stimulated with PMA in the absence of rottlerin. In particular, the organized redistribution of the F-actin was lost upon rottlerin pretreatment and the actin cytoskeleton appeared highly disorganized (Figure 4B).

Since it can be argued that rottlerin does not solely act through PKC-δ we employed the δC2 domain in a dominant negative strategy to interfere specifically with the PKC-δ–actin interaction. As shown in Figure 3, F-actin assumes a cortical localization in microinjected neutrophils. Although this localization is different from the localization in intact neutrophils shown in Figure 4, it is identical to that observed when neutrophils are analysed under conditions in which cell spreading is reduced at 20 °C (results not shown). Under these conditions, PMA treatment of intact cells leads to the redistribution of F-actin to one side of the cell.

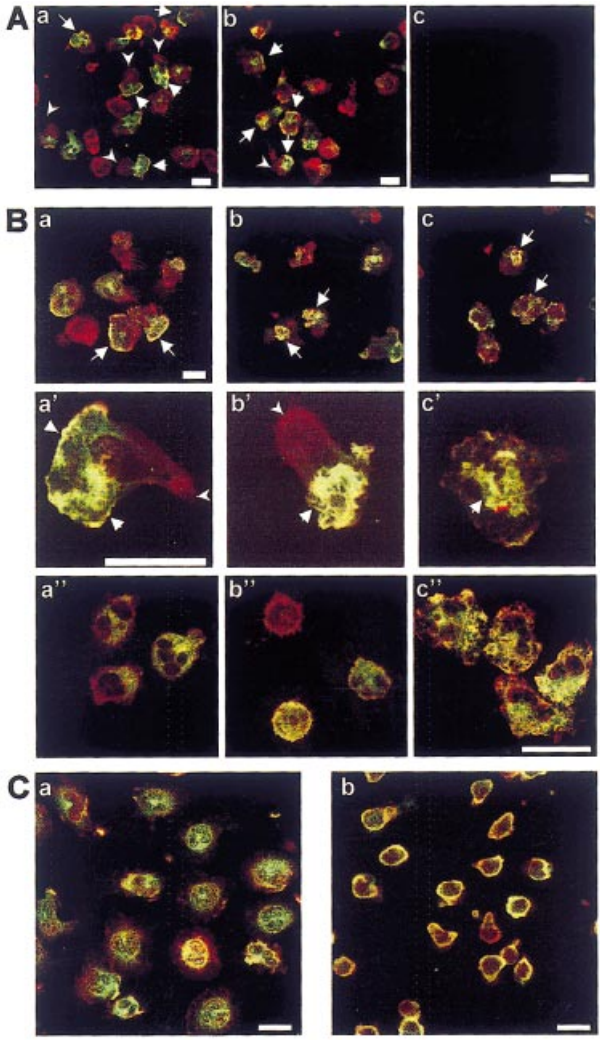

Figure 4 PKC-δ colocalizes with F-actin

Neutrophils were seeded on glass or fibronectin/collagen-coated coverslips and stained for PKC-δ and F-actin as indicated in the Materials and methods section. (**A**) Overlay of the PKC-δ signal (green) and the F-actin signal (red) in resting neutrophils attached to glass (**a**) or fibronectin/collagen (**b**) surfaces. Overlap of PKC-δ and F-actin is shown in yellow. Arrows indicate lamellipodia-like structures where colocalization of PKC-δ and F-actin was apparent. Arrowheads indicate the trailing end of neutrophils where F-actin, but no PKC-δ, was present. In samples incubated in the absence of primary anti-(PKC-δ) antibody and TRITC-phalloidin, no signal was observed (**c**). No bleeding occurred between channels. (**B**) Neutrophils where seeded on glass coverslips and activated with solvent (**a**–**a"**), IgG-opsonized *S. aureus* (**b**–**b"**) or 100 ng/ml PMA (**c**–**c"**) for 2 min, and the localization of PKC-δ and F-actin was determined. (**a**–**c**) Represent a projection of eight confocal sections, whereas (**a'**–**c'**) show a higher magnification of an individual cell from each condition. (**a"**–**c"**) Show neutrophils seeded on glass coverslips and preincubated for 30 min with 10 μM rottlerin after which they were stimulated as above. Arrows indicate areas of PKC-δ colocalizing with F-actin (yellow). Arrowheads indicate areas where F-actin, but not PKC-δ, was present. (**C**) Neutrophils were attached to glass for 5 min and incubated for 15 min with vehicle (**a**) or with 10 μM jasplakinolide (**b**) at 37 °C, then fixed and immunostained as above. PKC-δ (green), F-actin (red) and colocalization (yellow) are shown. Bar = 10 μm.

The same phenomenon was observed after PMA stimulation of microinjected cells, such that F-actin becomes localized to one side of the cell (Figure 5A). This effect was quantified by performing line scans through this apparent polarization axis for ten independently injected cells (Figure 5B). When δC2 was injected into the neutrophils, a complete inhibition of the PMA

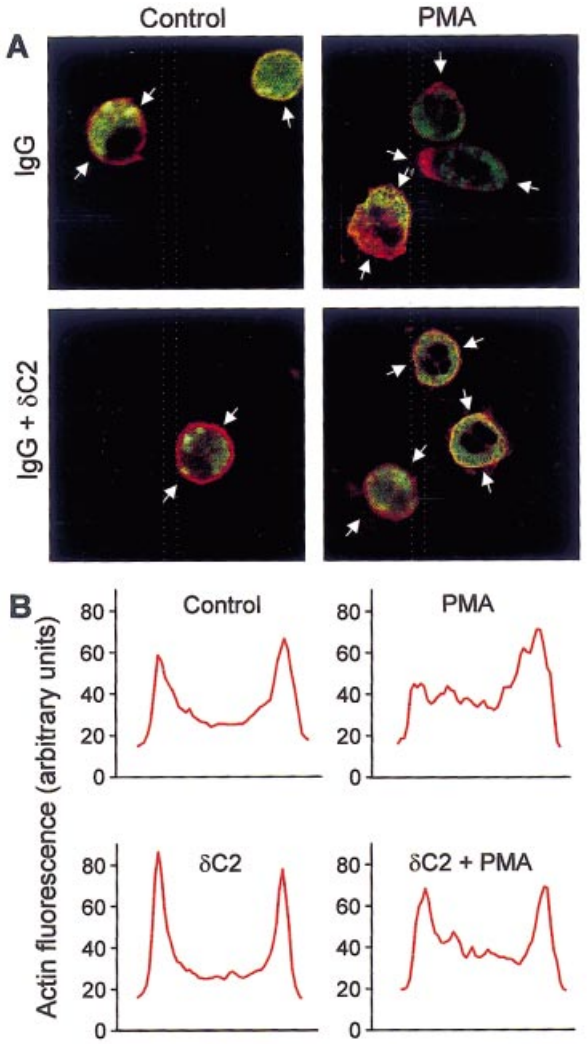

Figure 5 δC2 inhibits PMA-induced responses in microinjected cells

Cells were attached to fibronectin/collagen-coated coverslips and microinjected with rat IgG (IgG) with or without δC2. (**A**) After microinjection, cells were activated with PMA for 5 min, fixed and stained as described in the legend to Figure 3. F-actin was visualized using TRITC-phalloidin. (**B**) F-actin localization was quantified by measuring the red channel along an imaginary line across the cell [indicated by arrows in (**A**)]. In non-activated cells, the direction of the line was chosen randomly, whereas in PMA-activated cells the line was taken along the axis of polarization of the cell. The average F-actin distribution of ten cells taken from four independent microinjections is shown. Bar = 10 μm.

effect on actin was observed. Injection of the domain had no effect on F-actin in non-stimulated cells. Thus the presence of δC2 in neutrophils blocks PMA-induced changes in the actin cytoskeleton, indicating the involvement of PKC-δ in this process.

DISCUSSION

The present study indicates that the C2-like module, located in the PKC-δ N-terminal regulatory domain, interacts with β-actin. In intact neutrophils, PKC-δ colocalizes with F-actin in the flattened, leading cell end of control (migrating) cells, in the peripheral and apical ruffles on PMA-stimulated cells and in the area of particle intake in *S. aureus*-treated cells. Since these areas display a high degree of F-actin turnover, PKC-δ may play a role in this process. Evidence for this was obtained by interference with PKC-δ kinase activity, using a PKC-δ inhibitor, and by interfering with the interaction of PKC-δ and actin by microinjecting δC2. Altogether, our data confirm the suggestion that δC2 is a protein-interaction domain and indicate a function for PKC-δ in cytoskeletal F-actin regulation.

Our studies suggest that isolated δC2, as well as full-length PKC-δ, has the capacity to interact with F-actin. Application of jasplakinolide to neutrophils results in accumulation of PKC-δ in an F-actin-rich compartment. Jasplakinolide does not activate PKC-δ, but acts directly on actin, and PKC-δ redistribution is therefore secondary to F-actin accumulation. Thus in full-length PKC-δ, surface areas are exposed that allow interaction with F-actin. These surface areas are probably the δC2 domain, since in its isolated form this domain interacts directly with F-actin *in vitro*. PKC-δ may be present in a free form in the cytosol and become localized to F-actin upon jasplakinolide treatment. Alternatively, cellular PKC-δ may be complexed to G-actin and 'dragged along' upon accumulation of F-actin, or PKC-δ may be associated with a pool of F-actin that appears diffuse in normal cells [33] and that acts as source for F-actin accumulation upon jasplakinolide treatment.

In order to provide evidence that the interaction between F-actin and PKC-δ may be physiologically relevant we performed localization experiments. The localization of PKC-δ and F-actin in non-treated cells was not uniform, in fact in many cells significant polarization of both proteins was observed. Polarization of F-actin is a known property of many cell types, including HL-60 cells and neutrophils [36]; however, polarization of PKC-δ has not been shown before. Cellular polarization is associated with cell motility, in particular upon directional stimuli [37,38]. Under our control conditions no stimulus is presented, therefore the polarization of PKC-δ and F-actin may be part of a basal repertoire of explorative cellular behaviour. The application of PMA, which acts on PKC isotypes, including PKC-δ, results in a general redistribution of F-actin in many different directions, including a vertical direction. This is consistent with the observation by Downey et al. [9] who showed the appearance of ruffles on the surface of neutrophils after stimulation with PMA. Our results indicate that PKC-δ localizes to these ruffles, suggesting that it is a major candidate for mediating this PMA effect. Application of a directional stimulus, bacterial particles, has in principle the same effect: a redistribution of both F-actin and PKC-δ to the site of the stimulus.

In evaluating the localization of PKC-δ and F-actin it is important to note that not all PKC-δ colocalizes with cellular F-actin and that not all F-actin colocalizes with PKC-δ. Therefore the interaction between the two does not appear obligatory; indeed it seems to be confined to specialized sub-populations. Tertiary partners may be involved in these interactions. Such tertiary partners may bind to the C2 domain, or to other areas of PKC-δ. We have shown that the cytoskeletal protein GAP-43 binds to PKC-δ at the C2 domain [20]. GAP-43 is associated with the motile function of neuronal growth cones [39], although it is not clear at present whether this involves binding to PKC-δ. Because GAP-43 is a neuronal protein it is not a likely candidate for the specific localization of PKC-δ to the highly motile F-actin population in neutrophils; however, a GAP-43-like protein may play this role. Tertiary partners may also bind to PKC-δ outside the C2 domain. We have shown that a component of the NADPH oxidase in neutrophils, $p47^{phox}$, binds PKC-β and -δ at the catalytic domains [40], and that the NADPH oxidase enzyme system forms part of a cytoskeletal complex [41]. Evidence from Jaken and Parker [42] indicates that certain cytoskeletal PKC substrates interact, at least in part, through the pseudosubstrate site in the regulatory domain.

The fact that δC2, when microinjected into neutrophils, has inhibitory effects on actin rearrangements indicates a possible function for PKC-δ in the regulation of actin in these cells. It was described previously that stimulation of neutrophils with PMA resulted in actin rearrangements [9]. Using pharmacological manipulation, it was suggested that actin changes induced by phorbol esters required novel PKCs but not classical PKCs [9]. Our experiments, using δC2 in a dominant negative strategy employing the microinjection technique, make it highly likely that PKC-δ is the PKC isotype involved in these changes. By its nature this strategy provides the most specific way in which to interfere with PKC-δ. The mechanism involved is not clear, but is likely to involve the disruption of the interaction between PKC-δ and F-actin. Furthermore, additional circumstantial evidence for the regulation of actin by PKC-δ is based on the use of the PKC-δ inhibitor rottlerin, which we employed to investigate PKC-δ function in intact neutrophils.

Several studies indicate that PKC-δ is involved in actin regulation in other cells. In fibroblasts, PKC-δ acts on the actin cytoskeleton, affecting talin phosphorylation, disruption of microfilaments and recruitment of F-actin to the cell surface [43]. PKC-δ has also been linked to the formation of new focal adhesions in 3T3 fibroblasts [44], and to the regulation of αvβ5-dependent cytoskeletal associations and triggering tyrosine phosphorylation of focal adhesion kinase [45]. Also, its role in the assembly of focal adhesions during cell spreading on fibronectin has been suggested [46,47]. In apparent contrast with the present data are observations by Prekeris et al. [32] who showed that PKC-ε, but not PKC-δ, interacted with actin microfilaments. Prekeris et al. [32] used rabbit muscle actin as a source of actin for binding studies. We show that non-muscle actin binds the δC2 domain, but the domain failed to show significant binding to muscle actin. Thus our studies are in agreement on the lack of interaction of PKC-δ with muscle actin; however, our observations add to this in that PKC-δ interacts with β-actin.

In conclusion we have shown that PKC-δ interacts with F-actin through its C2-like domain. This interaction, probably in conjunction with other interactions, is important for the regulation of the F-actin cytoskeleton in neutrophils.

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