Specific isoforms of protein kinase C are essential for prevention of folate-resistant neural tube defects by inositol

Patricia Cogram, Andrew Hynes, Louisa P.E. Dunlevy, Nicholas D.E. Greene and Andrew J. Copp*

1Neural Development Unit, Institute of Child Health, University College London, London WC1N 1EH, UK

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A proportion of neural tube defects (NTDs) can be prevented by maternal folic acid supplementation, although some cases are unresponsive. The curly tail mutant mouse provides a model of folate-resistant NTDs, in which defects can be prevented by inositol therapy in early pregnancy. Hence, inositol represents a possible novel adjunct therapy to prevent human NTDs. The present study investigated the molecular mechanism by which inositol prevents mouse NTDs. Activation of protein kinase C (PKC) is known to be essential, and we examined neurulation-stage embryos for PKC expression and applied PKC inhibitors to curly tail embryos developing in culture. Although all known PKC isoforms were detected in the closing neural tube, use of chemical PKC inhibitors identified a particular requirement for ‘conventional’ PKC isoforms. Peptide inhibitors offer selective inhibition of individual PKCs, and we demonstrated isoform-specific inhibition of PKC in embryonic cell cultures. Application of peptide inhibitors to neurulation-stage embryos revealed an absolute dependence on the activity of PKCβ1 and γ for prevention of NTDs by inositol, and partial dependence on PKCζ, whereas other PKCs (α, βII, δ, and ε) were dispensable. To investigate the cellular action of inositol and PKCs in NTD prevention, we examined cell proliferation in curly tail embryos. Defective proliferation of hindgut cells is a key component of the pathogenic sequence leading to NTDs in curly tail. Hindgut cell proliferation was stimulated specifically by inositol, an effect that required activation of PKCβ1. Our findings reveal an essential role of specific PKC isoforms in mediating the prevention of mouse NTDs by inositol.

INTRODUCTION

Neural tube defects (NTDs) are common, severe birth defects with a multifactorial aetiology involving both genetic and environmental factors. In a proportion of cases, NTDs can be prevented by maternal peri-conceptional supplementation with folic acid (1,2). Around 30% of NTDs appear unresponsive to folic acid, however, and there is currently no preventive therapy available for these ‘folate-resistant’ defects. A large number of mouse genetic mutants exhibit NTDs and, as in humans, some of these are responsive to folic acid treatment whereas others are not (3,4). Mice homozygous for the curly tail mutation develop spina bifida that exhibits many similarities to the corresponding human NTD (5). NTDs in curly tail mice are folate-resistant (6), but we showed that the majority of NTDs in curly tail mice can be prevented by both myo-inositol and its D-chiro-enantiomer, either administered to pregnant females or directly to embryos in vitro (7,8). Our findings raise the possibility of developing a novel clinical therapy based on inositol. The recent observation that inositol therapy is well tolerated during human pregnancy at high risk of NTD recurrence (9), encourages progress towards a clinical trial of inositol.

The mechanism of action of inositol in preventing NTDs in curly tail mice involves activation of PKC, a family of serine/threonine kinases (7). The effect of inositol can be mimicked by brief treatment of embryos with TPA (12-O-tetradecanoylphorbol-13-acetate), an agonist of PKC. Moreover, the effect of inositol is blocked by co-administration of a broad-spectrum chemical inhibitor of PKC (7). At least 10 isoforms of PKC are known, grouped according to their dependence on diacylglycerol (DAG) and Ca2⁺ for activation (10). Conventional PKCs...
Among the cPKCs, only PKC\(\varepsilon\), PKC\(\eta\), or PKC\(\theta\) are weak or undetectable in the cytoplasmic fraction at E9.5 and with stage and cellular fraction (Fig. 1). cPKCs (\(\alpha\) isoforms could be detected, but their relative abundance varied which PKC isoforms are activated (i.e. associated with cytoplasmic (C), membrane-associated (Ps) and insoluble (Pi) isoforms are expressed in mouse embryos undergoing development in curly tail embryos (11), an effect that is blocked by inhibition of PKC\(\beta I\). Moreover, treatment of cultured cells with either the PKC activator TPA or inositol itself, could induce translocation of PKC isoforms, including PKC\(\beta I\) and \(\gamma\) to the nucleus. We conclude that PKC\(\beta I\) and \(\gamma\) are essential components of the molecular mechanism underlying the preventive effect of inositol on mouse NTDs.

**RESULTS**

**PKC isoform expression during mouse embryogenesis**

In order to identify the isoforms that might play a role in prevention of NTD, we initially determined which PKC isoforms are expressed in mouse embryos undergoing organogenesis (E9.5 to E16.5). Western blot analysis of cytoplasmic (C), membrane-associated (Ps) and insoluble (Pi) fractions of embryonic cell extracts indicated the degree to which PKC isoforms are activated (i.e. associated with membrane or insoluble cellular compartments). All PKC isoforms could be detected, but their relative abundance varied with stage and cellular fraction (Fig. 1). cPKCs (\(\alpha\), \(\beta\), \(\gamma\)) were weak or undetectable in the cytoplasmic fraction at E9.5 and E10.5, although expression increased from E11.5 onwards. Among the cPKCs, only PKC\(\gamma\) was present in the Ps fraction from E11.5 and in the Pi fraction at the latest stages. nPKCs (\(\delta\), \(\varepsilon\), \(\theta\), \(\eta\)) were expressed throughout the developmental period in both C and Ps fractions, whereas expression in the Pi fraction was more variable. aPKCs (\(\lambda\), \(\zeta\) and the related PKC\(\mu\)) were abundant from E9.5 onwards in all cellular fractions, with a fairly constant level of expression. All PKC isoforms were also detected in mutant curly tail embryos with no consistent differences in abundance compared with non-mutant CD1 mice (data not shown). Hence, the DAG-responsive cPKCs and nPKCs, which are likely to mediate the normalizing effect of inositol (6), exhibit evidence of stage-dependent activation unlike the aPKCs which appear ubiquitous and constitutively activated, as also described in other systems (12).

Western blot analysis was insufficiently sensitive to detect PKC isoform expression in specific embryonic regions, such as the posterior neuropore (PNP), the site of neural neurulation (13). We performed immunohistochemistry on histological sections through the PNP region, and detected expression of PKCs \(\alpha\), \(\beta I\), \(\beta II\), \(\gamma\) and \(\varepsilon\) in the closing neural tube, hindgut, notochord and presomitic mesoderm, at both E9.5 and E10.5 (Fig. 2). We conclude that although cPKCs, in particular, are variably expressed in whole neurulation-stage embryos by immunoblotting, they can be detected in all tissues of the PNP region by immunohistochemistry and, therefore, are candidates for a role in mediating the preventive effect of inositol on NTDs. Immunostaining was not carried out for the remaining isoforms as they were clearly detectable by western blot (Fig. 1).

**Chemical PKC inhibitors block the protective effect of inositol on NTDs**

We used PKC inhibitors to block the effect of inositol on neural tube closure. Curly tail embryos were cultured from E9.5 to E10.5, and the length of unclosed neural folds at the PNP was then measured to indicate predisposition to spina bifida (13). Embryos exposed to PBS (vehicle) alone had enlarged PNPs (Fig. 3A), reflecting the \(\text{in vivo}\) development of spinal NTDs by 50–60% of curly tail embryos (5), whereas treatment with myo-inositol decreased PNP length significantly (first grey bar, Fig. 3A), to a value characteristic of normally developing embryos (5). Hence, inositol normalizes PNP closure \(\text{in vivo}\), confirming previous findings (7,8). Curly tail embryos were exposed in culture to chemical inhibitors that block different combinations of PKC isoforms. Bisindolylmaleimide I (BisI) inhibits PKC\(\zeta\), \(\beta I\), \(\beta II\), \(\gamma\) and \(\varepsilon\) (14), Go6976 inhibits solely cPKCs (15), HBDDE inhibits PKC\(\zeta\) and \(\gamma\) \(\text{in vitro}\) (16) and LY294002 is an inhibitor of phosphatidylinositol-3-kinase (17), an activator of PKC\(\epsilon\) (18). When added alone, none of the inhibitors significantly altered PNP length (white bars, Fig. 3A). Moreover, BisV, an inactive control, did not prevent the reduction in PNP length caused by co-administration of inositol (second grey bar, Fig. 3A). In contrast, BisI, Go6976, HBDDE and LY294002 all partially blocked the normalizing effect of inositol, such that PNP length was reduced to a significantly lesser degree than in the presence of inositol alone (black bars, Fig. 3A). Hence, use of chemical inhibitors highlights the
potential importance of PKCα, β, γ, ε and ζ in mediating NTD prevention by inositol.

**Peptide inhibitors identify a critical role for PKCβI, γ and ζ in the inositol effect**

Because of the relatively broad spectrum activity of chemical PKC inhibitors, we next applied isoform-specific peptide PKC inhibitors to cultured curly tail embryos, to more precisely define the PKC isoform requirement for the inositol effect. Each peptide inhibitor was designed to the amino acid sequence corresponding to the isoform-specific RACK (receptor for activated C kinase) binding site on PKC. Inhibitors of this type prevent PKC translocation and/or activation in an isoform-specific manner (19,20). Peptide inhibitors were coupled to a sequence derived from the Drosophila Antennapedia homeodomain, ensuring cell permeability.

As with the chemical inhibitors, peptide PKC inhibitors alone had no significant effect on PNP length. Moreover,
the normalizing effect of inositol on PNP length persisted in the presence of inhibitors for PKC\(\alpha\), \(\beta I\), \(\delta\) and \(\epsilon\), as well as an inactive peptide (Fig. 3B). Strikingly, however, the co-addition with inositol of inhibitors for PKC\(\beta I\) or \(\gamma\) largely abolished the normalizing effect of inositol (black bars in Fig. 3B). Inhibition of PKC\(\zeta\) also partially blocked the normalizing action of inositol. These findings are consistent with the results from use of the chemical inhibitors, and indicate that the cPKCs, \(\beta I\) and \(\gamma\), and additionally PKC\(\zeta\), are critical for mediating the preventive effect of inositol.

**Activation of PKC isoforms in embryonic mouse cells is blocked by peptide inhibitors**

One possible explanation for the apparently isoform-specific PKC requirement for inositol action is that \(\beta I\), \(\gamma\) and \(\zeta\) are the only isoforms whose activation is blocked by peptide inhibitors in curly tail embryonic cells. To test this idea, primary fibroblastic cell cultures, prepared from caudal regions of E9.5 curly tail embryos were serum-starved, and then treated with the PKC agonist, TPA, in the presence or absence of peptide PKC inhibitors. Prior to TPA treatment, each PKC isoform exhibited cytoplasmic localization (Fig. 4A, D, G, J and M) but, following TPA treatment, all isoforms translocated to the nucleus (Fig. 4B, E, H, K and N), indicative of activation. In every case, addition of the appropriate peptide inhibitor blocked this TPA-induced translocation indicating that PKC activation had been prevented (Fig. 4C, F, I, L and O). Closely similar results were obtained with the mouse 3T3 cell line (data not shown).

In view of the close relationship between PKC\(\beta I\) and its alternatively spliced variant PKC\(\beta I\), we tested whether abrogation of the inositol effect by the PKC\(\beta I\) inhibitor might have resulted from antagonism of both PKC\(\beta I\) and \(\beta I I\) activation. PKC\(\beta I\) translocated to the nucleus following TPA stimulation, and this translocation was blocked by the \(\beta I\) inhibitor, but not by the \(\beta I I\) inhibitor (Fig. 5A–C). Conversely, translocation of PKC\(\beta I I\) was blocked by the \(\beta I I\) inhibitor but not the \(\beta I\) inhibitor (Fig. 5D–F). This finding confirms that the peptide inhibitors of PKC\(\beta I\) and \(\beta I I\) are indeed isoform-specific and suggests separate roles for these splice variants. We conclude that the requirement for specific PKC isoforms is unlikely to reflect differential activity of the peptide inhibitors, but may reflect a difference in participation of PKC isoforms in the action of inositol in preventing mouse NTDs.

To determine whether the PKC stimulation observed after TPA treatment also follows inositol administration, we studied PKC localization after inositol treatment. Inositol treatment was capable of inducing the translocation of PKC\(\alpha\) from the cytoplasm, as in serum-starved cells, to the nucleus (Fig. 5G and H). Similarly, PKC\(\beta I\) and \(\gamma\) isoforms whose activity is essential for prevention of NTD, were also found to exhibit nuclear localization in inositol-treated but not in untreated cells (data not shown). No difference in the localization of PKC\(\zeta\) could be detected after 6 h inositol treatment (50 \(\mu g/\mu l\)), but we cannot rule out the possibility that PKC\(\zeta\) is activated at a different dose or at a different time point. This observation suggests that PKC\(\alpha\), \(\beta I\) and \(\gamma\) can be activated by inositol treatment. However, PKC\(\alpha\) is not required for prevention of NTD whereas PKC\(\beta I\) and \(\gamma\) are essential.

**Embryonic cell proliferation is stimulated by inositol, in a PKC\(\beta I\)-sensitive manner**

Delayed closure of the PNP in curly tail embryos results from a reduced rate of cell proliferation, specifically in the embryonic hindgut, within the caudal region (3,11,21,22). The resulting proliferation imbalance causes ventral curvature of the caudal region, counteracting the apposition of the neural folds, and leading to spina bifida (23,24). The effect of inositol on cell proliferation was examined by immunohistochemistry for PCNA (proliferating cell nuclear antigen) and phospho-histone H3, markers of S-phase and M-phase of the cell cycle respectively. A significant stimulatory effect of inositol on
the percentage of PCNA-positive (Fig. 6A) and histone H3-positive (Fig. 6B) cells was detected in the hindgut, whereas inositol treatment had no effect on either proliferation marker in the neuroepithelium or notochord (Fig. 6A and B). Co-administration of the PKCβI peptide inhibitor blocked the stimulatory effect of inositol on hindgut proliferation, whereas the PKCε inhibitor had no effect. Neither inhibitor significantly altered proliferation in neuroepithelium or notochord. Hence, inositol overcomes the genetically-determined defect of cell proliferation in *curly tail* embryos, an effect that requires activation of PKCβI.

**DISCUSSION**

Inositol normalizes neural tube closure in the spinal region of *curly tail* mouse embryos destined to develop spina bifida (7), whereas folic acid has no preventive effect (6). Brief treatment with the PKC agonist TPA mimics the inositol effect, whereas the PKC inhibitor BisI abrogates inositol-mediated prevention (7). Here, we used other chemical PKC inhibitors, with reportedly narrower spectra of action, and showed that these are also able to block the inositol effect. However, the precise specificity of chemical PKC inhibitors may vary between cell types. For example, HBDDE is specific for PKCα and γ in vitro (16), but acts on other molecular targets in cerebellar granule neurons (25). This prompted the use of peptide PKC inhibitors which were able to block the translocation of specific PKC isoforms. Isoform-by-isoform analysis using these inhibitors revealed a requirement for PKCβI, γ and ε, but not other PKCs in the normalization of neural tube closure in *curly tail* embryos.
Developmental regulation of PKC isoform expression

We detected all PKC isoforms, during mouse development from E9.5 to E17.5, in agreement with previous analysis of PKC’s in E15–17 fetuses (26). Our findings contrast with a study of E10.5 embryos that detected PKCz, δ and ζ, but not βI, βII, γ and ε (27). This difference may reflect the low abundance of cPKCs on western blot, at early stages (E9.5–11.5) in particular, although all isoforms were clearly detectable in the PNP region by immunohistochemistry. Each PKC isoform exhibited a distinct distribution between subcellular fractions, which likely reflects the degree of activation. For instance, cPKCs were confined to the cytoplasmic fraction, suggesting minimal activation during undisturbed development, whereas aPKCs were present in membrane-associated and insoluble fractions throughout development, suggesting constitutive activation, as described in other systems (12). This finding is consistent with a role for the cPKCs, βI and γ, in mediating the preventive effect of inositol, since activation of these isoforms may be limiting in the absence of inositol, providing capacity for activation after treatment.

PKC isoforms and cell cycle regulation

We have demonstrated that inositol stimulates cell proliferation in the hindgut of curly tail embryos, reversing the imbalance of cell proliferation that is known to lead, via enhanced ventral curvature of the caudal region, to delay or failure of PNP closure (11,23,24,28). Moreover, PKCβI but not PKCα is required for this stimulation of cell proliferation. PKCβI has been previously associated with increased cell cycle entry. Its over-expression leads to increased proliferation of aortic endothelial cells, in contrast to PKCα which has an inhibitory effect (29). Similarly, proliferation of vascular smooth muscle cells is stimulated by PKCβI, through increased S-phase entry, but inhibited by PKCβII, through extension of S-phase (30). S-phase entry/progression has been suggested to be defective in hindgut cells of curly tail embryos (11), raising the possibility that activation of PKCβI may reverse this defect, leading to ‘rescue’ of the mutant phenotype. Interestingly, inositol treatment of cells for 6 h resulted in nuclear translocation of PKCβI and γ in some cells, as observed for short-term exposure to TPA. This nuclear localization suggests that a role of PKCβI in cell cycle regulation is plausible. PKCα also exhibits nuclear translocation in inositol-treated cells (both curly tail and non-mutant), but does not play an essential role in the prevention of NTD.

Molecular basis of the requirement for PKCζ and PI3-kinase

The normalizing effect of inositol on PNP closure was partially blocked by inhibition of PKCζ or PI3-kinase. The requirement for PKCζ may reflect a role in regulating other PKC isoforms (31). PKCζ is activated downstream of PI3-kinase (18), whose activity in turn depends on availability of phosphatidylinositol species. Hence, inositol administration may activate both PI3-kinase and PKCζ, by increasing flux through intracellular phosphoinositide metabolism. Indeed, inhibition of the inositol phosphate cycle by lithium blocks the preventive effect of inositol (7). The requirement for PI3-kinase may also be explained by its participation, with 3-phosphoinositide-dependent protein kinase-1 (PDK1) (32), in phosphorylation of the T-loop of the kinase domain, a requirement for activity of PKCs (33). Although we detected nuclear translocation of PKCζ, βI and γ following treatment of cells with inositol for 6 h, altered localization of PKCζ was not detected. This could be simply due to suboptimal dose or period of treatment (activation could be short-lived), but it is also likely that PKCζ activation occurs by a different mechanism to the classical isoforms as PKCζ is not diacylglycerol-responsive. Moreover, the partial requirement for PKCζ in the protective effect of inositol may reflect a permissive role for PKCζ activity that does not involve translocation to the nucleus.

Is PKC implicated in the mechanism of neural tube closure?

Several lines of evidence suggest that spinal neurulation per se does not depend on PKC activation and that defective closure in curly tail embryos is unlikely to result from an intrinsic deficiency in specific PKC isoforms. First, exposure of curly tail embryos to PKC inhibitors, in the absence of inositol, did not affect PNP closure. Similar findings were obtained with non-mutant CD1 embryos (data not shown). Second, there is no difference in abundance of PKC isoforms between affected and unaffected curly tail embryos (data not shown). Third, uptake and incorporation of 3H-inositol occurs similarly in cultured curly tail and non-mutant embryos, while inositol deficiency in vitro does not increase the penetrance of spinal NTDs in curly tail embryos (7). Finally, mice with targeted inactivation of the genes encoding PKCζ, β, γ, δ, ε and ζ do not exhibit neurulation defects (34–39). We suggest, therefore, that prevention of NTDs in curly tail mice by exogenous inositol operates via stimulation of the cell cycle in the embryonic hindgut, an effect that requires activation of specific PKC isoforms. Inositol is also reported to prevent NTDs in animal models of diabetes (40), whereas inositol deficiency causes cranial NTD, even in non-mutant mice (41). It remains to be determined whether the protective action of inositol also involves stimulation of cell proliferation in these cases.

MATERIALS AND METHODS

Mouse strains and embryo culture

Curly tail mice were maintained as described (5). Non-mutant random-bred CD1 mice were purchased from Charles River, UK. Mice were paired overnight and females checked for copulation plugs the following morning, designated embryonic day (E) 0.5. Embryos were explanted at E9.5 and those with 17–19 somites were cultured for 24 h in rat serum (6). Embryos were randomly allocated to treatment groups to minimize the effect of litter-to-litter variation. Cultures were supplemented with 1% (v/v) additions of phosphate buffered saline (PBS) or myo-inositol (50 μg/ml, final concentration). PKC inhibitors were added as 1% (v/v) additions. Bisindolylmaleimide (Bis) I and V, Go6976, HBDDE and LY294002 (all from Calbiochem) were diluted in minimal volumes of DMSO, further diluted in...
PBS, and added to cultures at final concentrations corresponding to their IC50 values (14–17): 10 μM (BisI, BisV, Go6976), 50 μM (HBDDE) or 1.4 μM (LY294002). DMSO diluted in PBS was added to control cultures. Peptide PKC inhibitors (supplied by Dr D. Mochly-Rosen) were added to embryo cultures to final concentrations of 1 μM: PKCaC2-4, PKCBIV5-3, PKCBIIIV5-3, PKCγC2-4, PKCaV1-1; PKCaV1-2 and PKCζ (pseudosubstrate region) (19,42–45). An Antennapedia peptide dimer served as an inactive control.

Statistical analysis

Comparison of multiple experimental groups was carried out by one way analysis of variance or by Kruskal–Wallis test (for data that were not normally distributed). Where significant variation was detected, multiple pairwise comparisons were performed by Tukey t-test or by Dunn’s test. A value of P < 0.05 was considered significant where the power of the test exceeded 0.8. Statistical tests were computed using SigmaStat 2.03 software.

Western blot of PKC isoforms

Embryos were homogenized in ice cold buffer (60 mM Tris/HCl, pH 7.5, 0.25 M sucrose, 10 mM EGTA, 5 mM EDTA, 500 μM leupeptin, 1 mM PMSF, 0.1 U/ml aprotinin, 1 mM dithiothreitol, 100 mM sodium vanadate, 50 mM sodium fluoride) and prepared as sub-cellular fractions: centrifugation at 100 000 × g for 30 min yielded a supernatant (C fraction). The pellet was resuspended in homogenization buffer containing 1% Triton X-100, and spun at 40 000 × g for 15 min., yielding a second supernatant (Ps-fraction) and a pellet (Pi-fraction). Samples were western blotted with mouse monoclonal IgGs for specific PKC isoforms (Transduction Laboratories), diluted as: α, 1:1000; β, 1:250; γ, 1:5000; δ, 1:500; ε, 1:1000; γ1/λ, 1:250; μ, 1:250; θ, 1:250; z, 1:1000, or rabbit polyclonal anti-PKC η (1:2000; Sigma). Constant protein loading was confirmed in parallel blots probed with rabbit anti-β-actin (1:5000; Sigma). Blots were exposed to horseradish peroxidase-linked rabbit secondary antibodies and visualized by ECL detection system (Amersham Bioscience). Positive controls were C-fraction from adult male CD1 mouse brain or spleen.

Immunohistochemistry

E9.5–10.5 embryos were fixed in 4% paraformaldehyde (PFA), embedded in paraffin wax and sectioned at 6 μm. Sections were re-fixed in 4% PFA then exposed to rabbit IgG primary antibodies to PKCζ, β, βII, γ or ε (1:200; Santa Cruz Biotechnology), anti-PCNA (1:100; Santa Cruz Biotechnology) or anti-phosphohistone H3 (1:100; Upstate Biotechnology). After washing in PBS, 0.1% BSA, 0.05% Triton X-100, sections were exposed to FITC-linked secondary antibodies (1:40; Jackson Immuno Research). Controls included non-specific rabbit IgG [R&D Systems, 1:100 dilution in 10% foetal calf serum (FCS) in PBS] in place of primary antibody or pre-absorption of primary antibody with blocking peptides. Both yielded no signal. Labelling indices (number of labelled cells/total cell number × 100) were obtained from alternate transverse sections through the rostral end of the PNP (i.e. the region of maximal neural fold elevation) stained for PCNA or histone H3. Experiments were carried out in triplicate by an observer blinded to treatment type.

Primary embryonic cell cultures

The trunk distal to the forelimb bud was removed from 15 curly tail embryos (average stage: 27 somites), disrupted mechanically and dissociated by 20 min incubation at room temperature in F10 medium containing 0.12% w/v sodium bicarbonate, 10 mM HEPES, pH 7.4, 1 mg/ml trypsin, 1.5 mg/ml collagenase. After trituration, the suspension was passed through a Nitex filter and centrifuged for 5 min at 1000g. Pelleted cells were resuspended in 5 ml growth medium (Dulbecco’s Modified Eagle’s Medium, 100 units/ml penicillin, 100 μg/ml streptomycin) containing 10% v/v FCS, then seeded onto tissue culture dishes and cultured at 37°C in 5% CO2. For experiments, 105 cells (passage 5) were seeded onto glass cover slips coated with poly-L-lysine and fibronectin, cultured for 5 h in growth medium containing 10% FCS, and transferred to growth medium containing 1% FCS for 18 h (“serum starvation”). Cells received either: (i) 100 mM TPA, 10 min prior to fixation in 4% PFA; (ii) 100 mM isoform-specific peptide PKC inhibitor, 20 min prior to fixation, then 100 mM TPA, 10 min prior to fixation; (iii) 50 μg/ml myo-inositol, 6 h prior to fixation; or (iv) no treatment. For analysis of the effect of inositol, parallel experiments were carried using 3T3 cells. Cells were processed for immunohistochemistry as above and analysed in triplicate by an observer blinded to treatment type.

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