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Protein kinase C activation by 12–0-tetradecanoylphorbol 13-acetate in CG-4 line oligodendrocytes stimulates turnover of choline and ethanolamine phospholipids by phospholipase D and induces rapid process contraction

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Abstract

Treatment of [^{3}H]-choline- or [^{14}C]-ethanolamine-labelled undifferentiated bipolar and differentiated multipolar CG-4 line oligodendrocytes with 12–0-tetradecanoylphorbol 13-acetate (TPA) to activate protein kinase C stimulated the release of choline or ethanolamine metabolites to the medium over controls. Ro31–8220, a PKC inhibitor, reduced TPA-stimulated release of choline- and ethanolamine-metabolites to basal levels. TPA treatment of both bipolar and multipolar cells caused rapid contraction of processes leaving rounded up cells: this effect was blocked by Ro31–8220. After 12–15 h exposure to TPA, bipolar undifferentiated CG-4 line cells extended short processes again and the cells became multipolar. Nocodozole, an agent which disrupts microtubules and caused CG-4 line cells to round up, caused increased choline or ethanolamine-metabolite release to the medium over basal levels suggesting that some release during TPA-treatment might occur due to process fragmentation. However, the transphosphatidylation reaction confirmed that phospholipase D was active in these cells. Exposure of bipolar undifferentiated CG-4 line cells to TPA resulted in down-regulatation of PKC-α and PKC-β which could not be detected by Western blotting after a few hours; PKC-ε was down-regulated much more slowly but PKCs δ, ζ and ι were not influenced by 48 h exposure of cells to TPA. Formation of phosphatidylethanol in the transphosphatidylation reaction was markedly reduced in TPA down-regulated cells indicating a role for PKCs α and β in phospholipase D activation in CG-4 line oligodendrocytes.

Keywords: CG-4 line oligodendrocytes, protein kinase C subspecies α and β_{II}, phospholipase D, choline and ethanolamine phospholipid hydolysis, transphosphatidylation, process contraction.

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Phospholipase D (PLD) hydrolyses phosphoglycerides to phosphatidic acid (PtdOH) and the polar head group (Kiss 1996; Rhee and Dennis 1996; Exton 1997, 1999; Waite 1999). The PtdOH so formed may function as a signalling molecule and/or may also be converted to diacylglycerol (DAG) perhaps to sustain activation of protein kinase C (Nishizuka 1992). In support, Ha and Exton (1993) have found that DAG derived from PLD action in IIC9 fibroblasts can activate PKC-ε but not PKC-α. However, in aortic endothelial cells stimulation of PLD does not lead to activation of PKC (Pettitt *et al*. 1997). The role of PtdOH and DAG as intracellular messengers has been discussed by Hodgkin *et al*. (1998). Lyso-PtdOH, an intercellular lipid messenger with growth factor-like activity (Moolenaar *et al*.

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Abbreviations used: C/M, chloroform : methanol; DAG, diacylglycerol; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethylsulphoxide; FBS, fetal bovine serum; MARCKS, myristoylated alanine-rich C-kinase substrate; OLs, oligodendrocytes; PIP2, phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; PLD, phospholipase D; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdEtOH, phosphatidylethanol; PtdOH, phosphatidic acid; TPA, 12–0-tetradecanoylphorbol 13-acetate.

1997) which can also influence cell shape (Manning *et al*. 1998), can also be generated from PtdOH. PtdCho is the main lipid species hydrolysed when PLD is activated in cells (Waite 1999) but there is evidence in fibroblasts (Kiss *et al*. 1999) and especially in glial cells (McNulty *et al*. 1992; Kiss 1996; Van Iderstine *et al*. 1996; Morreale *et al*. 1997), that PLD activation results in the turnover of PtdEtn as well as of PtdCho. Van Iderstine *et al*. (1996) have shown in C6 glioma cells that both plasmalogenic and diacyl ethanolamine phosphoglycerides are hydrolysed by PLD.

Two separate PLD genes (PLD1 and PLD2), approximately 50% identical, have been defined in humans: PLD1 and PLD2 require PIP2 for activity (Frohman *et al*. 1999). Activation of PLD1, but not PLD2, is regulated partly through the protein kinase C (PKC) family of phospholipid-dependent serine/threonine kinases (Exton 1999): treatment of cells with phorbol ester analogues of diacylglycerol activates the transphosphatidylation reaction and the release of free choline and ethanolamine indicative of PLD activity (Kiss 1996; Exton 1997). These phorbol ester effects can be blocked by inhibitors of PKC including staurosporin derivatives such as Ro31–8220 (Van Iderstine *et al*. 1996; Morreale *et al*. 1997). The α and β subspecies of PKC are implicated in PLD activation by phosphorylation-dependent and phosphorylation-independent mechanisms (Exton 1999; Houle and Bourgoin 1999). Other pathways to activation of PLD involve small G proteins such as Rho and ARF (Exton 1999). A fatty acid activated PLD activity independent of phosphoinositides and not activated by PKC has also been described (Frohman *et al*. 1999). Activation of PLD in astrocytes in response to phorbol ester and a range of agonists is well defined (for example, Brunner and Murphy 1990; Gustavsson *et al*. 1993) but little is known about PLD in precursor or mature oligodendrocytes. In a human oligodendroglioma line carbachol and histamine, but not glutamate or bradykinin activate PLD (Dawson *et al*. 1993).

Here we have investigated whether the oligodendrocyte model cell line CG-4 which can be cultured as a bipolar precursor OL or a multipolar differentiated cell form, contains PLD activity linked to PKC. We have found that PKC activation by TPA stimulates turnover of both choline and ethanolamine phosphoglycerides in these CG-4 line OLs and that this is due to activation of PLD as indicated by the transphosphatidylation reaction. We have also observed that phorbol ester treatment of bipolar undifferentiated CG-4 line OLs causes rapid contraction of cell processes and a release of MARCKS protein to the cytosol. After about 12–15 h exposure to TPA, cells extend processes again but have a multipolar, differentiated morphology. PKC-α and β are rapidly down-regulated in CG-4 line cells by TPA treatment: these PKC subspecies are linked to PLD activation since their down-regulation results in a reduced formation of PtdEtOH in the transphosphatidylation reaction.

Materials and methods

Materials

Culture flasks were from Life Technologies (Glasgow, UK). Twenty-four-well plates were from Iwaki (SLS Ltd, Nottingham, UK). Dulbecco's modified Eagle's medium (DMEM), antibiotics and trypsin/EDTA (10×) were from GibcoBRL (Life Technologies, Glasgow, UK). Fetal bovine serum was from PAA Laboratories (Kingston upon Thames, UK). Chemicals and media supplements for N1 medium were from Sigma unless otherwise stated. TPA and 4α-phorbol were from Alexis Corp. (Nottingham, UK). Ro31–8220 was a gift from Dr G. Lawson, Roche Discovery (UK). [^{14}C]-ethanolamine hydrochloride (NEC038) and [^{3}H]-choline chloride (NET109) were from NEN (Hounslow, UK).

Cell culture

B104 neuroblastoma cells were grown in DMEM containing 10% fetal bovine serum with antibiotics. N1 medium was DMEM supplemented with insulin (5 μg/mL), transferrin (50 μg/mL), putrescene (100 μM), progesterone (20 nM), selenium (30 nM) and biotin (40 nM) with antibiotics plus bovine serum albumin (1.25 mg/mL) as we have found this aids cell growth. B104 cell-conditioned medium was prepared by twice rinsing B104 cells at 80% confluency with DMEM and adding 10 mL N1 medium per 75 cm^2 flask. This medium was recovered after 3 days, centrifuged to remove any debris and stored at − 20°C. N1 medium was mixed 70 : 30 (v/v) with B104-conditioned medium and then filter sterilized. Rat CG-4 line progenitor oligodendrocytes (from Dr J. C. Louis, California, with permission), passsage 40–50, were cultured on poly-L-lysine-coated plates in N1/B104 medium to ensure a bipolar morphology (Rumsby *et al*. 1999). CG-4 line cells were passaged at 80–90% confluency while bipolar by rinsing with Tris-saline and releasing with trypsin/EDTA (Gibco) for 6 min at 37 °C. DMEM/10% FBS was added to inhibit the trypsin and cells were collected by low speed centrifugation. They were resuspended in N1/B104 medium and passaged 1 : 3 into fresh poly-L-lysine-coated flasks or into poly-L-lysine-treated 24-well plates for experiments. Differentiated CG-4 line cells were grown in N1 medium + 0.5% fetal bovine serum.

Phospholipid metabolite release

For lipid metabolite release, 2.5×10^4 CG-4 line OLs in appropriate medium were seeded into wells of a poly-L-lysine-coated 24-well plate and allowed to settle and extend processes for 24 h in the presence of 0.2 μCi/well [^{3}H]-choline chloride and/or [^{14}C]-ethanolamine hydrochloride in 0.5 mL N1/B104 medium. We have shown previously that equilibrium labelling of C6 glioma cell PtdCho and PtdEtn by the approach used occurs within 15–20 h (McNulty 1991) and so is likely to be complete for CG-4 line OLs by the 24-h period used in these experiments. Further, variation in cell number between wells after 24 h in culture varies by about 10% or less (McNulty and Rumsby, unpublished). The labelling medium was then removed and cells were rinsed twice with fresh N1 or N1/B104 medium warmed to 37 °C. A final fresh 0.5 mL aliquot of prewarmed medium containing TPA, Ro31–8220, TPA + Ro, DMSO or 4α-phorbol as appropriate at the concentrations noted in the text was then added and a 50-μL aliquot immediately removed for measurement of time zero

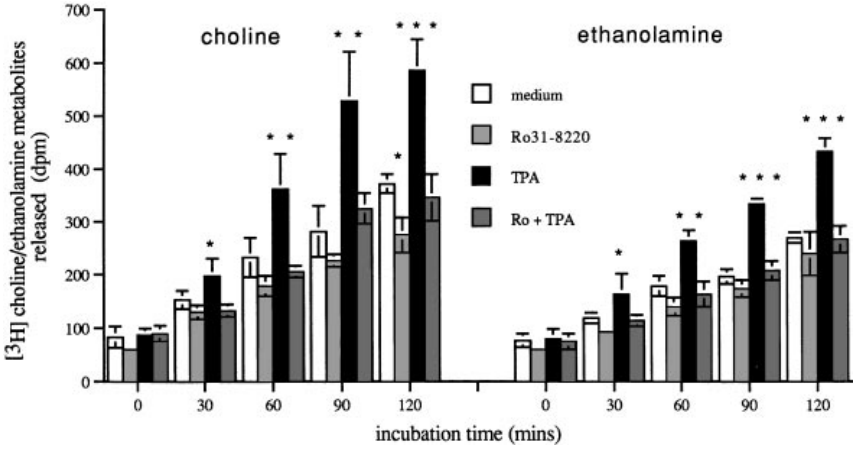

Fig. 1 TPA treatment of bipolar undifferentiated CG-4 line oligodendrocytes stimulates a release of choline and ethanolamine lipid metabolites to the medium over a 2-h time course. Bipolar CG-4 line cells were radiolabelled for 24 h as described in the Methods section. Labelling medium was removed and cells rinsed several times with fresh warm medium. Finally, 500 μL fresh warm medium was added containing TPA (100 nM) or Ro31–8220 (1 μM) as appropriate. Release of radioactive lipid metabolites to the medium was measured as described. Results are mean dpm released/20 μL aliquot medium ± SD ($n = 6$) from a typical experiment. Stimulated choline and ethanolamine metabolite release values at 30 min and beyond are significantly different from basal values ($^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). Inhibition of basal choline metabolite release by Ro31–8220 alone only becomes significant at the 120-min time point ($^*p < 0.05$).

radioactivity in the medium. Further 50 μL aliquots of medium were then recovered from the same wells at the time intervals described. Aliquots of medium were centrifuged at full speed in an Eppendorf centrifuge to pellet any membrane debris and duplicate 20 μL aliquots taken for scintillation counting in 2 mL Ultima Gold XR (Packard, Pangbourne, UK) keeping samples from the same wells in sequence. For ethanolamine metabolite release experiments the final medium used on cells contained 5 mM cold ethanolamine hydrochloride. DMEM contains choline so extra was not added in release experiments. Results are means from three or four separate wells with aliquots of medium counted in duplicate and are ± standard deviations. In some experiments time zero counts are shown (e.g. Fig. 1). Where time zero counts are not shown values have been subtracted from the time points shown. Phase contrast examination of cells was on a Nikon Optiphot microscope and photomicrographs were taken on T-max 100 film (ASA 200).

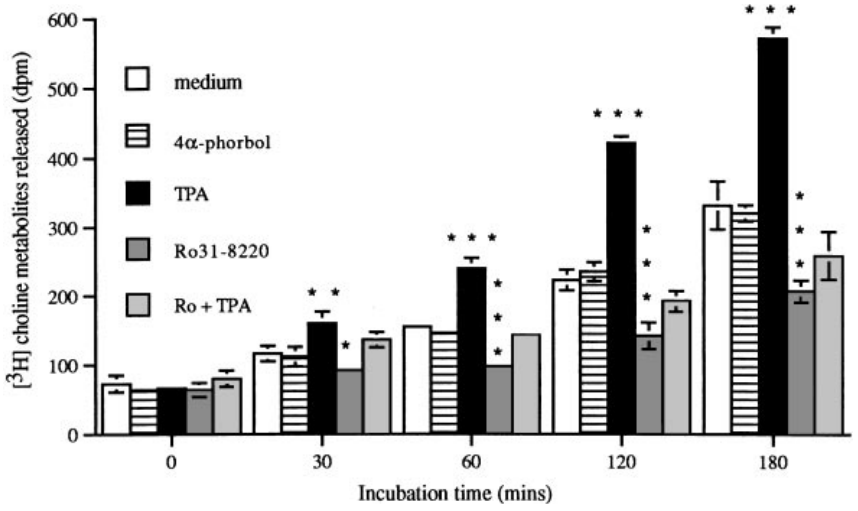

Fig. 2 TPA treatment of differentiated multipolar CG-4 line oligodendrocytes stimulates a release of choline lipid metabolites to the medium over a 3-hour time course. Bipolar CG-4 line cells were differentiated for 24 h in N1 medium during which time they were labelled with [^{3}H]-choline. Cells were then treated as described in the Methods section with TPA (100 nM), the inactive 4α-phorbol or Ro31–8220 (1 μM) to measure stimulated lipid metabolite release. Release of radioactive lipid metabolites to the medium was measured as described. Results are mean dpm released/20 μL aliquot medium ± SD ($n = 6$) from a typical experiment. Stimulated choline metabolite release values at 30 min and beyond are significantly different from basal release values ($^{**}p < 0.01$, $^{***}p < 0.001$). Inhibition of basal choline metabolite release by Ro31–8220 alone is significant throughout the time course ($^*p < 0.05$, $^{***}p < 0.001$).

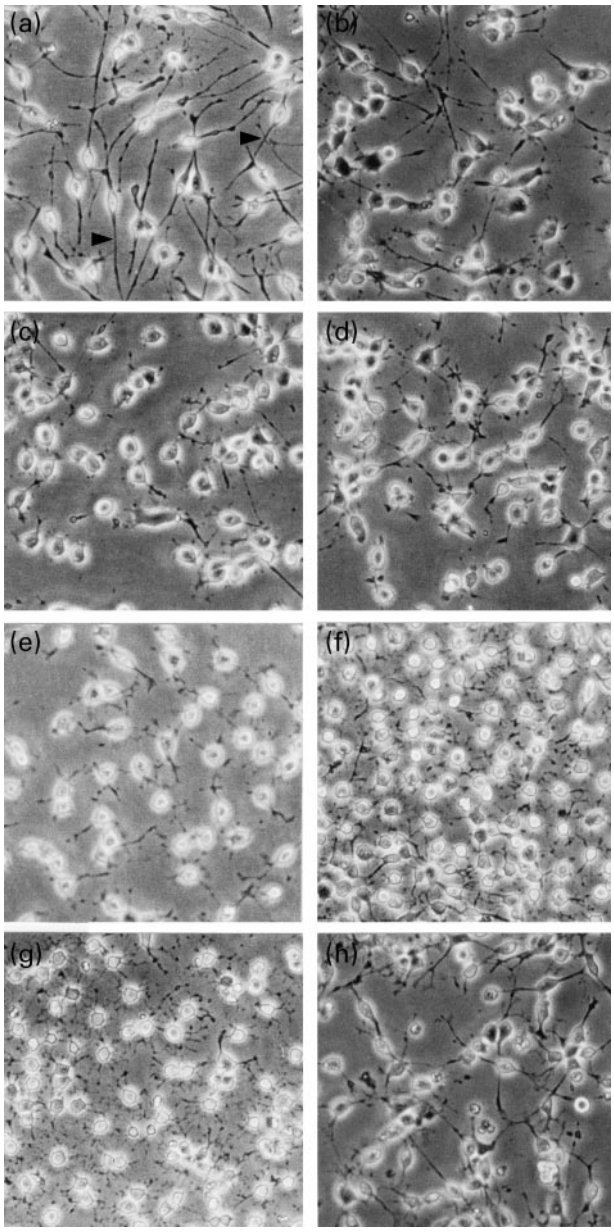

Fig. 3 Changes in morphology induced by treatment of undifferentiated bipolar CG-4 line oligodendrocytes with TPA. Bipolar CG-4 line cells in poly-L-lysine-coated 24-well plates in N1/B104 medium were treated with 100 nM TPA or TPA + Ro31–8220 (1 μM). Changes in morphology were recorded by phase contrast photomicroscopy at times up to 48 h. (a) Normal untreated bipolar CG-4 line OLs, B-G, TPA-treated cells (b, 1 h; c, 2 h; d, 6 h; e, 12 h; f, 24 h; g, 48 h). (h) TPA + Ro31–8220-treated cells after 2 h. Mag × 380.

Transphosphatidylation reaction

Cells in 6-well plates were incubated with 0.5 μCi/well [^{3}H]-myristate for 4 h to label mainly a PtdCho pool but some PtdEtn is also labelled. The labelling medium was removed, cells rinsed with fresh warm medium and 1 mL fresh medium containing 1% ethanol added. Cells were then stimulated with DMSO or 4α-phorbol, TPA or TPA + Ro31–8220 for 3 h. Medium was then removed and cells were rinsed with cold Tris-saline and allowed to drain free of rinse medium on ice. Lipids were extracted by treating cells in wells twice with 2 mL 1 : 2 (v/v) chloroform : methanol (C/M) and then twice with 2 mL 1 : 1 (v/v) C/M. Extracts were pooled, evaporated to dryness, redissolved in 100 μL C/M 2 : 1 (v/v) containing authentic phosphatidylethanol and phosphatidic acid (Lipid Products Ltd, Surrey, UK) and 50 μL applied to thin-layer chromatography plates (Keiselgel H slurried in 80 mM potassium oxalate). Plates were developed in a solvent system of chloroform : methanol : acetic acid (9 : 1 : 1, v/v), allowed to air dry and PtdEth and PtdOH located by iodine vapour staining. Lipid spots were marked, plates stood overnight and adsorbent scrapped into scintillation vials with 3 mL scintillant as above. Experiments were conducted in triplicate. Results are mean PtdEth or PtdOH dpm ± SD.

Western blotting

Western blotting to detect PKC subspecies and MARCKS in bipolar CG-4 line oligodendrocytes was undertaken as described elsewhere (Drew *et al.* 1996). In PKC down-regulation experiments bipolar CG-4 line cells were treated in 25-cm^2 flasks with 250 nM TPA or 4α-phorbol. Cells were harvested into homogenization buffer as described for analysis of PKC subspecies at defined time intervals. Homogenates were assayed for protein using the BCA method (Pierce, UK) prior to addition of 10% sodium dodecyl sulfate (SDS) and sample buffer, heating at 100°C for 5 min and resolution by SDS-PAGE on 10% gels followed by western blotting. In down-regulation/transphosphatidylation experiments, cells were first treated with TPA for 6 h to down-regulate PKC-α and were then labelled with [^{3}H]-myristate for 4 h. Cells were then rinsed, fresh medium containing 1% ethanol added followed by TPA (100 nM) to further activate PKC. Reactions were stopped after 2 h by rinsing wells with cold Tris-saline, allowing wells to drain and extracting total lipids as described above for resolution of PtdEtOH.

Statistical treatment

Where appropriate, results have been compared by Student's *t*-test and are considered to be significantly different when $p < 0.05$.

Results

Undifferentiated CG-4 line cells passaged in N1/B104 medium into poly-L-lysine-coated wells settled immediately and rapidly extended processes to adopt a bipolar morphology as we have shown elsewhere (Rumsby *et al.* 1999). These undifferentiated bipolar CG-4 line cells expressed the early progenitor oligodendrocyte markers A2B5 and GD3 (Louis *et al.* 1992).

Unstimulated [^{3}H]-choline-labelled undifferentiated bipolar CG-4 line cells slowly released radiolabelled choline metabolites to the medium in the 2-h period studied as shown in Fig. 1 (choline): levels of radioactivity detected in the medium sampled at time zero were very similar. This

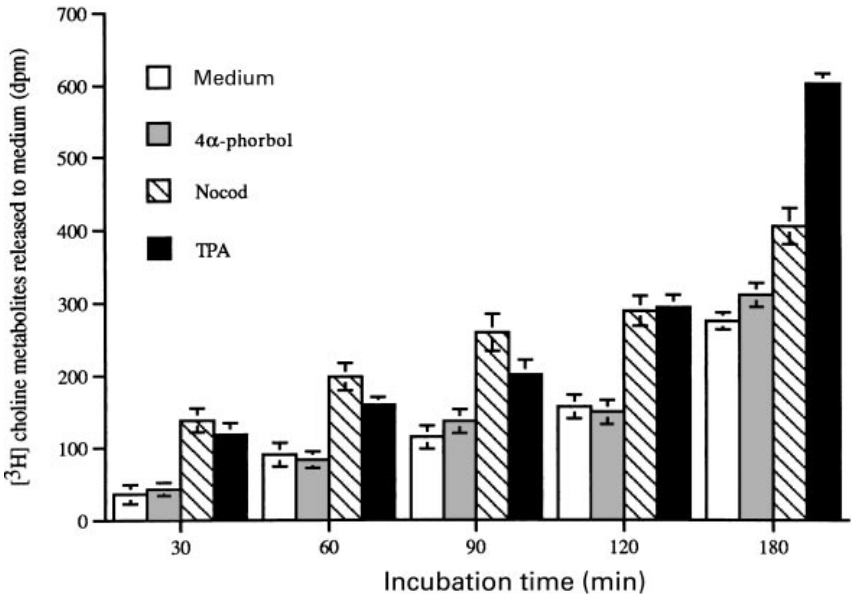

Fig. 4 Effect of nocodozole damage to processes on [^{3}H]-choline metabolite release from bipolar CG-4 line oligodendrocytes relative to TPA. Cells in 24-well plates were labelled as described and then rinsed. Fresh medium containing 4α-phorbol (100 nM), nocodozole (5 μM) or TPA (100 nM) was added and aliquots removed for measurement of radioactivity at 60-, 120- and 180-min intervals. Results are mean dpm/20 μL aliquot medium ± SD ($n = 6$). Mean time zero dpm have been subtracted throughout.

basal release of choline metabolites is due to the normal turnover of phosphatidylcholine in cells growing in N1/B104 medium without stimulation. In separate experiments we showed that treatment of [^{3}H]-choline-labelled CG-4 line cells with medium containing DMSO vehicle or 100 nM 4α-phorbol, an inactive phorbol which does not activate PKC, was similar to that observed with medium alone (results not shown). Treatment of labelled cells with 100 nM TPA to activate PKC stimulated the release of radiolabelled choline metabolites to the medium; this stimulation was observed within 30 min of adding the phorbol ester (Fig. 1, choline) and was significantly different from unstimulated release to the medium at this time and beyond. In separate experiments we showed that this TPA-stimulated release continued for over 3 h, as did basal release into medium alone (data not shown). With C6 glioma cells the TPA-stimulated release of choline metabolites continues above basal values for up to about 20 h by which time values match those of basal release due to normal turnover of phospholipid headgroups: maximum release of radiolabel occurs after about 6 h (Skippen and Rumsby, unpublished results). We did not establish if choline metabolite release from CG4 line OLs follows this same longer term trend as the purpose of the experiment was to look at PLD activation. The PKC inhibitor Ro31–8220 alone, at 1 μM, had no stimulatory effect on choline-metabolite release (Fig. 1, choline). In fact, Ro31–8220 alone seemed to have a slight inhibitory effect on basal choline metabolite release but this was only significant at the 120-min time point. Then 1 μM Ro31–8220 added with

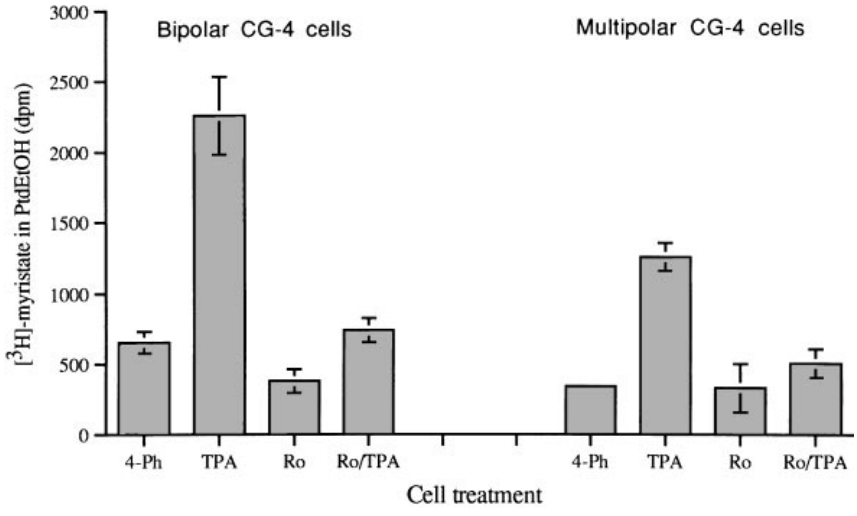

Fig. 5 The transphosphatidylation reaction indicates that PLD is active in undifferentiated and differentiated CG-4 line oligodendrocytes. Undifferentiated and differentiated CG-4 line cells were cultured in triplicate in poly-L-lysine-coated 6-well plates. Cells were labelled for 4 h with [^{3}H]-myristate. Fresh medium containing 1% ethanol was added and cells were stimulated as shown. After 3 h cells were rinsed with cold Tris-saline and lipids extracted for PtdEtOH analysis as described. Results show mean dpm per PtdEtOH spot from a thin-layer plate and are ± SD ($n = 3$).

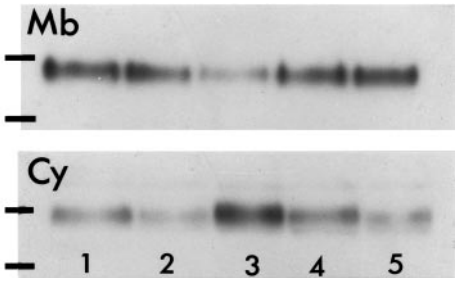

Fig. 6 Translocation of MARCKS protein from membrane (Mb) to cytosol (Cy) on TPA treatment of bipolar CG-4 line oligodendrocytes and reassociation with the membrane after 40 min. Undifferentiated CG-4 line cells were stimulated with 100 nM TPA, 100 nM 4α-phorbol or TPA + 1 μM Ro31–8220. After 20 and 40 min cells were rinsed, scraped from flasks, homogenized and 100 000 g membrane (M) and cytosol (C) fractions recovered for western blotting as described. 1, untreated cells, 2, 4α-phorbol-treated cells, 3, TPA, 20 min, 4, TPA, 40 min, 5, TPA + Ro31–8220. Equal protein (30 μg) loadings. Markers are 97 and 78 kDa.

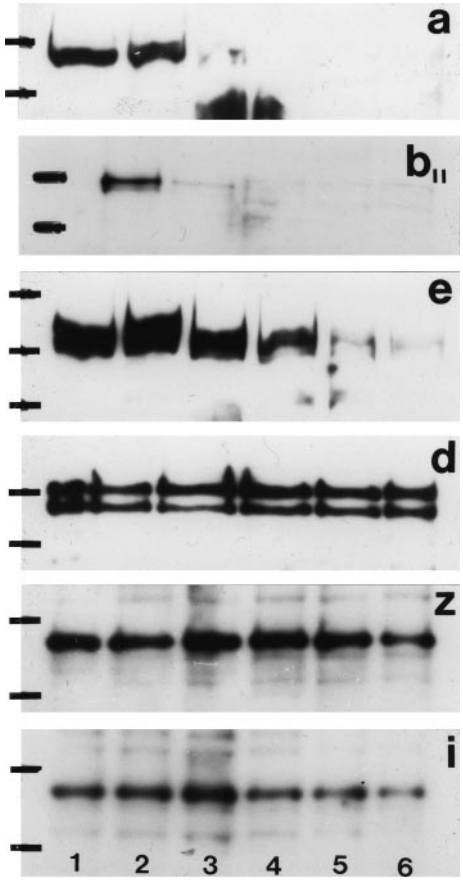

Fig. 7 Down-regulation of PKC subspecies in bipolar CG-4 line oligodendrocytes by long-term TPA treatment. Cells were treated with 100 nM TPA and harvested at the time intervals shown for western blotting for PKC subspecies α (a), β_{II} (b_{II}), ε (e), δ (d), ζ (z) and ι (i) as described. lane 1, untreated cells; lane 2, TPA 2 h; lane 3, TPA 6 h; lane 4, TPA 12 h; lane 5, TPA 24 h, lane 6, TPA 48 h. Equal protein (30 μg) loadings applied. Markers: 97 kDa and 78 kDa, (e) 135, 97 and 78 kDa.

100 nM TPA completely abolished the effect of the phorbol ester in stimulating choline metabolite release.

Treatment of [^{14}C]-ethanolamine-labelled bipolar CG-4 cells with 100 nM TPA to activate PKC stimulated the turnover of ethanolamine phosphoglycerides as shown by the increased release of ethanolamine metabolites to the medium compared with unstimulated cells (Fig. 1, ethanolamine). TPA stimulated release became significant at time points of 30 min and beyond. DMSO vehicle and 4α-phorbol (results not shown) gave release results similar to medium alone. As with choline-labelled cells, 1 μM Ro31–8220 had no stimulatory effect on ethanolamine-metabolite release but did reduce the TPA-stimulated release of ethanolamine metabolites to basal levels. Ro31–8220 had no significant effect inhibiting basal release of ethanolamine metabolites at the 2 h time point.

Differentiated multipolar CG-4 line cells labelled with [^{3}H]-choline or [14C]-ethanolamine also showed a TPA stimulated release of lipid headgroup metabolites to the medium (Fig. 2, ethanolamine results not shown). As with undifferentiated bipolar cells the TPA effect was inhibited by Ro31–8220. Alone, this inhibitor had no stimulatory effect on lipid metabolite release from differentiated multipolar CG-4 line OLs (Fig. 2). As with its effect alone on bipolar CG-4 line cells, Ro31–8220 had a significant inhibitory effect reducing basal choline metabolite release at 30-min time points and especially later. The results presented in Figs 1 and 2 show the reproducibility of the TPA effect on choline and ethanolamine metabolite release from both bipolar and multipolar forms of the CG-4 oligodendrocyte cell line.

The morphology of normal undifferentiated bipolar CG-4 line cells and the morphological changes which occur on phorbol ester treatment are shown in Fig. 3. Normal bipolar undifferentiated CG-4 line cells have long thin processes extended on either side of the small cell body (Fig. 3a, arrows). Treatment of these cells with 100 nM TPA rapidly induced process retraction and loss. Within 30–60 min most cells had lost their fine bipolar morphology and remnants of contracted/fragmented processes remained (Fig. 3b). By 2 h many cells had lost processes completely and were rounded up: this effect was complete within 2–3 h (Fig. 3c). By 6–12 h (Figs 3d and e) cells were starting to extend short, more stubby processes and later after 24–48 h TPA treatment they developed a multipolar morphology (Figs 3f and g). Addition of 1 μM Ro31–8220 largely inhibited this TPA-induced fragmentation of processes (Fig. 3h), whereas cells treated with 100 nM TPA + 1 μM Ro31–8220 for 120 min resemble untreated cells. In preliminary experiments we have found that differentiated multipolar CG-4 line oligodendrocytes also lose their shorter processes when treated

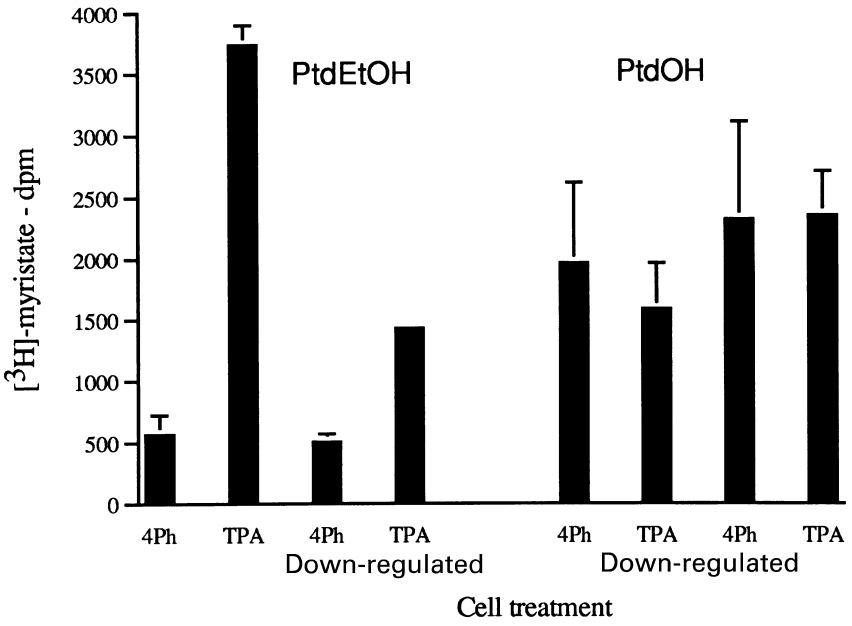

Fig. 8 Downregulation of PKC-α and PKC-β_{II} in bipolar CG-4 line oligodendrocytes decreases the formation of PtdEtOH in the transphosphatidylation reaction. Bipolar CG-4 line cells were treated with 250 nM TPA for 6 h and were then labelled with [^{3}H]-myristate for another 4 h. Control cells were just labelled with [^{3}H]-myristate for 4 h. Cells were rinsed and fresh medium containing 250 nM 4α-phorbol (4Ph) or 250 nM TPA with 1% ethanol was added. After 3 h cells were rinsed, lipids extracted as described and PtdEtOH resolved by TLC. Results shown are mean dpm/PtdEtOH or PtdOH spot from a TLC plate ± SD ($n = 3$).

with 100 nM TPA. As with bipolar CG-4 line cells, this effect is inhibited by 1 μM Ro31–8220 (results not shown).

The possibility that the observed TPA-stimulated release of choline and ethanolamine metabolites from CG-4 line cells arises by leakage as processes contract or fragment was examined by treating [^{3}H]-choline-labelled cells with 5 μM nocodozole (Fig. 4) an agent which disrupts microtubules and in doing so causes bipolar CG-4 line cell processes to fragment and the cells to round up. Results are shown with time zero dpm subtracted. CG-4 line cells treated with 5 μM nocodozole rapidly lost processes and after 3 h treatment had rounded up and resembled TPA-treated cells. Over this time period nocodozole treatment caused an increase in choline metabolite release to the medium over basal and 4α-phorbol control cell values (Fig. 4). Choline metabolite release caused by nocodozole was about the same as that observed by treatment with 100 nM TPA. This nocodozole effect was observed from 30 to 120 min treatment, and by 120 min choline metabolite release from TPA- and nocodozole-treated cells was virtually the same. However, by 3 h when cells had fully rounded up, the effect caused by nocodozole was much reduced compared with that observed with TPA treatment. These findings with nocodozole suggest that some lipid metabolite release possibly could arise from process damage at early times but that by later time points lipid metabolite release is caused chiefly by the stimulated turnover of PtdCho and PtdEtn by PLD activation by PKC.

The transphosphatidylation reaction was used to verify that PLD was being activated in both bipolar undifferentiated and multipolar differentiated CG-4 line OLs. Phorbol ester treatment of [^{3}H]-myristate-labelled bipolar or multipolar CG-4 line cells in the presence of ethanol stimulated the formation of PtdEtOH (Fig. 5) as revealed by an approximately threefold increase in radioactivity into PtdEtOH compared with 4α-phorbol-treated cells. The PKC inhibitor Ro31–8220 eliminated this effect (Fig. 5) reducing radioactivity in the PtdEtOH component to 4α-phorbol control values. Ro31–8220 alone at 1 μM did not stimulate PtdEth formation.

Results in Fig. 6 show that MARCKS protein immunoreactivity associated with total membranes of bipolar CG-4 line cells is decreased after 20 min TPA stimulation and is increased in a 100 000 g cytosol fraction when equal amounts of protein (30 μg) are resolved by SDS-PAGE and Western blotting. The results show surprisingly that by 40 min phorbol ester treatment MARCKS immunoreactivity has returned to the membrane fraction and is lost from the cytosol compartment. This finding was unexpected since, as shown in Fig. 3, process contraction continues to take place for some hours after this re-association of MARCKS protein with the membrane.

Western blotting results (Fig. 7) reveal that PKC-β_{II} immunoreactivity is lost rapidly when bipolar CG-4 line OLs are treated with TPA. PKC-α is also down-regulated by TPA treatment and immunoreactivity cannot be detected after about 6 h (Fig. 7). PKC-ε was down-regulated more slowly: some PKC-ε immunoreactivity was still detectable after 24 h. PKC-δ, PKC-ζ and PKC-ι were not down-regulated by TPA treatment even after 48 h treatment. Undifferentiated bipolar CG-4 line OLs were exposed to TPA for 6 h to down-regulate PKC-α and PKC-β_{II}. When these cells were then labelled for 4 h with (^{3}H)-myristate followed by restimulation with TPA in fresh medium

containing 1% ethanol in a transphosphatidylation reaction a reduction in the formation of PtdEtOH relative to 4α--phorbol treated cells was detected as shown in Fig. 8. Levels of PtdOH measured in 4α-phorbol and TPA-treated cells were not influenced by the down-regulation treatment. This may be because PtdOH can be generated by pathways other than via PLD which are not influenced by PKC, for example from diacylglycerol via DAG kinase.

Discussion

Knowledge of the structure of PLD and its activation by PKC or by small G proteins of the Arf and Rho families has advanced rapidly in the last few years (Exton 1999; Frohman *et al*. 1999; Houle and Bourgoin 1999). Phorbol ester stimulation of the transphosphatidylation reaction in a range of mammalian cells, indicative of PLD activity, demonstrates a PKC pathway to PLD activation which may involve phosphorylation-dependent and phosphorylation-independent mechanisms. In Swiss 3T3 cells and C6 glioma cells PKC-α is the subspecies linked to PLD activation: in Rat-6 fibroblasts PKC-β_I may be involved (Frohman *et al*. 1999). PKC activates PLD1, but not PLD2 or a fatty acid-activated PLD; both PLD1 and PLD2 are dependent on phosphoinositides for activity. PLD1 hydrolyses PtdCho exclusively in most mammalian cells but PLD activities acting on other phospholipids have been occasionally reported (Balsinde *et al*. 1989; Wang *et al*. 1991; Huang *et al*. 1992; Madesh and Balasubramanian 1997). In glial cells it seems well established that PtdEtn as well as PtdCho is hydrolysed by the PLD activity present (McNulty *et al*. 1992; Mallon 1996; Van Iderstine *et al*. 1996). In C6 glioma cells both plasmalogen and diacyl ethanolamine phosphoglycerides are degraded (Van Iderstine *et al*. 1996). We have shown previously that the bulk of the radioactivity released on TPA stimulation is in the form of free Cho or Etn indicative of PLD activation (McNulty *et al*. 1992; Mallon 1996). Radiolabelled Etn released to the medium in such experiments derives from PLD activity and is not formed by the action of a phosphatase on phosphoethanolamine arising by PLC activity (Van Iderstine *et al*. 1996).

Here we have examined PKC-stimulated PLD activity in CG-4 line OLs (Louis *et al*. 1992). In its undifferentiated and differentiated forms this CG-4 cell line retains some characteristics of 0–2 A progenitor glia (bipolar morphology, expression of A2B5 and GD3) and mature OLs (galactocerebroside and some myelin basic protein expression), respectively (Louis *et al*. 1992). Our present results indicate that in both undifferentiated bipolar and differentiated multipolar forms, CG-4 line OLs have TPA-activated PLD activity as shown by the transphosphatidylation reaction and that this PLD activity hydrolyses both PtdCho and PtdEtn as shown by the release of radiolabelled choline and ethanolamine metabolites to the external medium. The TPA effect on both PtdCho and PtdEtn is inhibited by Ro31–8220, a catalytic site inhibitor of PKC confirming the involvement of PKC in the activation of PLD. It has been reported that Ro31–8220 can inhibit p70 S6 kinase and MAPK-activated protein kinase-1β as well as PKC (Alessi 1997) but we found that the inhibitory effect of Ro31–8220 on the TPA-induced activation of PLD occurs by blocking PKC rather than via effects on these other kinases (Morreale *et al*. 1997). Ro31–8220 alone at concentrations up to 1 μM had no stimulatory effect on PtdCho or PtdEtn turnover unlike its far less specific parent compound staurosporine which stimulates PLD activity in C6 cells at concentrations over 1 μM (Van Iderstine *et al*. 1996). Of interest was the observation that Ro31–8220 alone has an inhibitory effect on the basal release of choline lipid metabolites in both bipolar and multipolar CG-4 line cells (Figs 1 and 2). This suggests that PKC, perhaps by activating PLD, is involved in regulating the basal turnover of PtdCho and PtdEtn in these cells.

Our present findings suggest that at least two forms of PLD may be expressed in these CG-4 line OLs. A PLD1 activity probably accounts for the stimulated turnover of PtdCho in the cells since PLD1 is activated by PKC and specifically hydrolyses PtdCho (Waite 1999). This means, however, that another PLD activity linked to PKC must catalyse the observed stimulated turnover of PtdEtn. PLD2 and a fatty acid-activated PLD are not linked to PKC (Frohman *et al*. 1999) so, even if expressed, will not be activated to account for the turnover of PtdEtn. Thus our results suggest that, as with C6 glioma cells, undifferentiated and differentiated CG-4 line OLs may express another PLD activity acting on ethanolamine phospholipids. In CG-4 line OLs the PLD1 activity present might hydrolyse both PtdCho and PtdEtn but this would conflict with existing findings in other cells (Hammond *et al*. 1997; Waite 1999). We have also observed that the TPA-stimulated turnover of PtdEth in C6 cells is sensitive to wortmannin, while TPA-stimulated turnover of PtdCho is not (Morreale *et al*. 1997). This supports the view that a separate PE-PLD activity is expressed in C6 cells but it remains to be seen whether wortmannin has a similar inhibitory effect on PKC-linked turnover of PtdEtn in CG-4 line OLs. The recent finding that PLD translocates on activation to the plasma membrane (Brown *et al*. 1998) suggests that PLD isozymes may have varied intracellular locations. Both membrane and cytosolic PLD activities have been reported (Wang *et al*. 1991); the cytosolic PLD activity hydrolysed PtdEtn and PtdIns as well as PtdCho while the membrane-associated activity only hydrolysed PtdCho. The localization of PLD activities in different cell compartments has been summarized by Liscovitch *et al*. (1999).

We found that TPA treatment of bipolar undifferentiated CG-4 line OLs induces a rapid down-regulation of PKC-β_{II}

and PKC-α. Cells treated with TPA for 10 h to down-regulate these two PKC subspecies had a markedly reduced formation of PtdEtOH in the transphosphatidylation reaction, leading us to conclude that PKCs α and/or β_{II} provide the major link to PLD activation in these cells. This is in agreement with findings on other cells where the α- and β-subspecies of PKC are linked to PLD activation (Frohman *et al*. 1999). A rat brain phospholipase D, showing 90% amino acid identity with human PLD 1b, is activated by PKC-α and PKC-β_{II} but not other PKC subspecies in an ATP-independent reaction (Min *et al*. 1998). Kiss *et al*. (1999) have recently reported that PKC-ε can inhibit phorbol ester-induced PLD activity via its regulatory domain indicating that the control of PLD activity through PKC is more complex than originally indicated.

TPA treatment induced rapid contraction of the long thin processes of bipolar undifferentiated CG-4 line cells. The processes initially developed a blebby appearance after TPA treatment and then fragmented. Within the time scale of experiments (2–3 h) many cells lost their processes completely (Fig. 2). There was thus the possibility that the observed release of radioactivity from TPA-treated cells arises by damage to processes rather than by PLD activity. To check this we treated [^{3}H]-choline-labelled bipolar CG-4 line cells with nocodozole which disrupts microtubules and causes process loss. At 1 and 2 h time points nocodozole treatment caused a slight release of radioactivity to the medium, similar to TPA, suggesting that at early time points some leakage of radioactivity as processes contract might contribute to the lipid metabolites detected in the medium. However, by 3 h a clear stimulation of choline metabolite release due to TPA activation of PKC, with a reduced nocodozole effect, is indicative of PLD activity. It is important to note that, while we used nocodozole to test for lipid metabolte release on process fragmentation, the mechanisms by which TPA and nocodozole cause process damage and cell rounding up are almost certainly different. Nocodozole is a microtubule disrupting agent, whereas the TPA effect is likely to be caused by changes in the actin cytoskeleton as shown by the effects observed on MARCKS protein as discussed below. Treatment of bipolar and multipolar CG-4 line OLs with cytochalasin D, which disrupts actin microfilaments, does not cause fragmentation of CG-4 line cell processes (Hughson, Afsari and Rumsby, in preparation).

TPA treatment causes cell rounding and loss of filopodia (Rosen *et al*. 1990), an effect correlated with phosphorylation of MARCKS, an actin cross-linking protein associated with maintenance of membrane–microfilament interactions (Aderem 1995). Our results show that MARCKS protein is expressed by bipolar undifferentiated CG-4 line OLs and that TPA treatment of cells for 20 min causes a release of MARCKS protein immunoreactivity from membranes to the cytosol. Such findings implicate MARCKS protein in process stability in bipolar CG-4 line OLs. Surprisingly, the results also reveal that MARCKS immunoreactivity is restored to a membrane association even though TPA is still present. This is surprising in view of the fact that process contraction and fragmentation continues for some hours. MARCKS is localized to processes with PKC-δ in bipolar 0–2 A progenitor glial cells (Moreton *et al*. 1995). The role of MARCKS protein in OL process stability is not straightforward, however, since we have noted that disruption of microfilaments in bipolar CG-4 line OLs by treatment with cytochalasin D does not cause fragmentation of processes (Hughson, Afsari and Rumsby, unpublished data). Further, activation of PLD in CG-4 line OLs following stimulation of PKC may result in the generation of lysoPtdOH (van Dijk *et al*. 1998), which can influence the cell cytoskeleton (Zigmond 1996) and may contribute to process damage.

PKC involvement in OL process extension is clearly rather complex. We show here that short-term activation of PKC induces process contraction and fragmentation. However, in mature OLs long-term phorbol ester treatment to activate PKC is reported to promote process outgrowth (Yong *et al*. 1988, 1991, 1994; Althaus *et al*. 1990). Phorbol ester activation of PKC causes $O4^+$ GC^- precursor OLs to transiently revert to a more immature phenotype with loss of O4 expression and a change to a bi- or tri-polar form (Avrossa and Pfeiffer 1993). After several days exposure to TPA, these cells reacquire the original phenotype and mature further. We seem to observe the same with CG-4 line OLs, at least morphologically, since after 12–15 h exposure to TPA, the cells re-extend short processes and adopt a mature multipolar morphology though they are not in differentiation medium, are still exposed to phorbol ester and have apparently down-regulated all PKC-α and PKC-β_{II}. It seems unlikely therefore that PKC-α can be contributing to mechanisms regulating the re-extension of processes which occurs in these CG-4 line cells after 15–24 h exposure to TPA.

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