

**Keywords:** Etn phosphoglycerides; phorbol ester; protein kinase C; ethanolamine; prostate cancer epithelial cell lines; phospholipase D

# Phorbol ester stimulates ethanolamine release from the metastatic basal prostate cancer cell line PC3 but not from prostate epithelial cell lines LNCaP and P4E6

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**Background:** Malignancy alters cellular complex lipid metabolism and membrane lipid composition and turnover. Here, we investigated whether tumorigenesis in cancer-derived prostate epithelial cell lines influences protein kinase C-linked turnover of ethanolamine phosphoglycerides (EtnPGs) and alters the pattern of ethanolamine (Etn) metabolites released to the medium.

**Methods:** Prostate epithelial cell lines P4E6, LNCaP and PC3 were models of prostate cancer (PCa). PNT2C2 and PNT1A were models of benign prostate epithelia. Cellular EtnPGs were labelled with [1-<sup>3</sup>H]-Etn hydrochloride. PKC was activated with phorbol ester (TPA) and inhibited with Ro31-8220 and GF109203X. D609 was used to inhibit PLD (phospholipase D). [<sup>3</sup>H]-labelled Etn metabolites were resolved by ion-exchange chromatography. Sodium oleate and mastoparan were tested as activators of PLD2. Phospholipase D activity was measured by a transphosphatidylolation reaction. Cells were treated with ionomycin to raise intracellular Ca<sup>2+</sup> levels.

**Results:** Unstimulated cell lines release mainly Etn and glycerylphosphorylEtn (GPEtn) to the medium. Phorbol ester treatment over 3h increased Etn metabolite release from the metastatic PC3 cell line and the benign cell lines PNT2C2 and PNT1A but not from the tumour-derived cell lines P4E6 and LNCaP; this effect was blocked by Ro31-8220 and GF109203X as well as by D609, which inhibited PLD in a transphosphatidylolation reaction. Only metastatic PC3 cells specifically upregulated Etn release in response to TPA treatment. Oleate and mastoparan increased GPEtn release from all cell lines at the expense of Etn. Ionomycin stimulated GPEtn release from benign PNT2C2 cells but not from cancer-derived cell lines P4E6 or PC3. Ethanolamine did not stimulate the proliferation of LNCaP or PC3 cell lines but decreased the uptake of choline (Cho).

**Conclusions:** Only the metastatic basal PC3 cell line specifically increased the release of Etn on TPA treatment most probably by PKC activation of PLD1 and increased turnover of EtnPGs. The phosphatidic acid formed will maintain a cancer phenotype through the regulation of mTOR. Ethanolamine released from cells may reduce Cho uptake, regulating the membrane PtdEtn:PtdCho ratio and influencing the action of PtdEtn-binding proteins such as RKIP and the anti-apoptotic hPEBP4. The work highlights a difference between LNCaP cells used as a model of androgen-dependent early stage PCa and androgen-independent PC3 cells used to model later refractory stage disease.

The plasma membrane of prostate cancer (PCa) cells provides a primary contact surface with basement membrane, other cancer cells and stroma as well as with immune cells. Malignancy alters the fatty acid composition of complex lipids in such membranes

driven by a switch to anabolic metabolic pathways (Suburu and Chen 2012). Prostate cancer cells and transformed prostate epithelial cell lines also contain elevated levels of choline (Cho) and ethanolamine (Etn) metabolites indicative of changes in

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phosphatidylcholine (PtdCho) and ethanolamine phosphoglyceride (EtnPG) turnover (e.g., Ackerstaff *et al*, 2001, 2003; Swanson *et al*, 2008; Komoroski *et al*, 2011) as reported for other cancer cells and transformed cell lines (e.g., Aboagye and Bhujwalla, 1999; Ackerstaff *et al*, 2001, 2003; Herminghaus *et al*, 2002; Iorio *et al*, 2005; Glunde *et al*, 2006; Elyahu *et al*, 2007; Podo *et al*, 2007). In PCa cells, levels of phosphorylEtn (PEtn) exceed those of phosphorylCho (Podo, 1999; Swanson *et al*, 2008; Komoroski *et al*, 2011). Further, the glycerylphosphorylEtn (GPEtn) to glycerylphosphorylCho ratio increases in PCa as with many transformed cells (Singer *et al*, 1995; Bell and Bhakoo, 1998; Podo, 1999; Ackerstaff *et al*, 2003; Swanson *et al*, 2008; Komoroski *et al*, 2011). Such changes in Etn metabolites reveal that turnover of EtnPGs, as well as that of PtdCho, is altered in tumorigenesis. Serum EtnPG levels are also raised in PCa (Zhou *et al*, 2012).

These changes in Cho and Etn metabolite levels in malignant cells occur because of the increased expression and/or activity of enzymes regulating PtdCho and EtnPG metabolism including Cho/Etn kinase (Cho/EtnK), PLD, PtdCho-specific phospholipase C (PtdCho-PLC) and phospholipase A2 (PLA<sub>2</sub>) (e.g., Ramirez de Molina *et al*, 2002, 2008; Foster and Xu, 2003; Foster, 2009; Iorio *et al*, 2010; Dong *et al*, 2010; Caiazza *et al*, 2011). Increased expression of Cho/Etn transporters and faster rates of Cho or Etn uptake (Katz-Brull *et al*, 2002; Elyahu *et al*, 2007; Mintz *et al*, 2008; Iorio *et al*, 2010) also contribute to altered phospholipid metabolite levels in transformed cells. Ethanolamine phosphoglycerides are substrates for PLD and their turnover by PLD increases on PKC activation indicating involvement of PLD1 (Kiss and Anderson, 1989; Hii *et al*, 1991; Kiss and Tomono, 1995). PtdEtn may even be an exclusive PLD substrate in some cell lines (Kiss *et al*, 1994). Because tumorigenesis influences PKC-stimulated PtdCho turnover in prostate epithelial cell lines (Rumsby *et al*, 2011), we have now investigated whether EtnPG turnover is linked to PKC and is also altered by tumorigenesis.

## MATERIALS AND METHODS

**Cell culture.** Benign prostate epithelial cell lines PNT2C2 and PNT1A and the cancer-derived cell line LNCaP were cultured in RPMI1640 with added glutamine, HEPES and 10% FBS (R10). The cancer-derived P4E6 cell line was cultured in keratinocyte serum-free medium with pituitary extract and epidermal growth factor additives and 2% FBS (K2A). The cancer-derived metastatic PC3 cell line was cultured in Hams F-12 with 7% FBS (F7). Passage numbers were: PNT2C2 < 150; PNT1A < 80; P4E6 < 50; LNCaP < 50; PC3 < 50. Cells were passaged by rinsing with Tris-saline, releasing with Tris-trypsin for 10 min at 37 °C and pelleting in R10 to inactivate trypsin followed by resuspension in their normal growth medium for continued growth, or in low serum medium (e.g., R2.5, F2.5, K2) or medium lacking serum for experiments. All cell lines were Mycoplasma-free and were genotyped (Powerplex 16, Promega, Southampton, UK; Cat. No. DC6531) to ensure identity and genomic stability.

**Effect of cell density on basal Etn metabolite release.** PNT2C2 cells were seeded (in triplicate) into 24-well plates at 2.5-, 5-, 7.5 × 10<sup>4</sup> and 1 × 10<sup>5</sup> in 0.5 ml R2.5 medium and labelled with 0.5 μCi [1-<sup>3</sup>H]-Etn chloride (American Radiolabelled Chemicals, Stevenage, UK) per well for 36 h. Labelling medium was removed, cells were rinsed once in warm R0 and then incubated at 37 °C for 60 min in R0. Cells were further rinsed twice with warm R0 to remove released [<sup>3</sup>H]-Etn-labelled metabolites. Finally, 0.5 ml R0 containing 1 mM unlabelled Etn and PEtn was added. Basal release of Etn metabolites was monitored by taking 30 μl aliquots of medium at T = 0, 1, 2 and 3 h for scintillation counting (below) and by replacing medium removed with fresh R0 to maintain

volume. After 3 h, remaining medium was centrifuged to pellet cell debris. Etn metabolites released from cells were resolved on ion exchange columns as below.

**[<sup>3</sup>H]-Etn metabolite release.** Cells (7.5 × 10<sup>4</sup>) were seeded in triplicate into 24-well plates in R2.5, F2.5 or K2 as appropriate and labelled with [1-<sup>3</sup>H]-Etn hydrochloride as above. LNCaP cells were cultured on poly-L-lysine-coated wells or amine plates (BD, Oxford, UK) to improve adhesion during rinses. Cells were used just sub-confluent to minimise changes due to contact inhibition or cell cycle effects. Labelled cells were rinsed as above and incubated with 0.5 ml serum-free medium containing 1 mM Etn hydrochloride and 1 mM PEtn hydrochloride (to minimise rapid [<sup>3</sup>H]-Etn reuptake) plus PKC activators/inhibitors (see Figure legends) per well (Rumsby *et al*, 2011). Thirty microlitre aliquots of medium were removed from wells at T = 0 and at times indicated in figures; fresh medium (+/- inhibitors) was added to wells to maintain volume. Aliquots were centrifuged at 13 000 r.p.m. to pellet any cell debris; duplicate 10 μl aliquots were then removed into Top Count plates for scintillation counting with 100 μl Microscint-20 (Perkin-Elmer, Beaconsfield, UK). Mean c.p.m. values from triplicate wells were calculated +/- s.d. (n = 6). At the end of experiments, media were centrifuged and frozen for later Etn metabolite analysis.

**Separation of Etn and Cho in medium.** Aliquots of media were spotted onto Kieselgel G TLC plates (Merck Millipore, Nottingham, UK) with standards of [1-<sup>3</sup>H]-Etn and [<sup>3</sup>H]-Cho in adjacent lanes. Plates were dried and developed in methanol (MeOH): 0.5% NaCl (1:1, v/v). After drying, lanes with standards and media were divided into nine 1.5-cm sections. Adsorbent in each section was scraped into scintillation vials with 2 ml Ultima Gold XR scintillant (Perkin-Elmer) to detect positions of standards indicating resolution of Etn and Cho in the media lanes.

**Separation of PtdEtn and PtdCho.** [1-<sup>3</sup>H]-Etn-labelled cells remaining from release experiments above were extracted once with 0.5 ml MeOH and twice with 0.5 ml chloroform (CHCl<sub>3</sub>):MeOH (1:2v/v) and 0.5 ml CHCl<sub>3</sub>:MeOH (1:1v/v) to recover total lipids. Solvent extracts were pooled and dried under nitrogen. Lipids were redissolved in CHCl<sub>3</sub>:MeOH (2:1v/v) and triplicate aliquots resolved by TLC on Kieselgel H plates in CHCl<sub>3</sub>:MeOH:H<sub>2</sub>O (5:2:1 v/v) against authentic PtdEtn and PtdCho standards (Lipid Products, Nutfield, UK). Positions of lipids were identified with iodine vapour. Appropriate areas of adsorbent containing PtdCho and PtdEtn were scraped into vials with 2 ml scintillant (as above) for scintillation counting.

**Resolution of GPEtn, PEtn and Etn.** Etn metabolites were resolved by ion exchange chromatography (Kiss *et al*, 1994). Radioactivity in triplicate 0.5 ml aliquots of each fraction was measured by scintillation counting to calculate total d.p.m./fraction. Sodium oleate (1 mM) in samples had no effect on the elution pattern of GPEtn, PEtn and Etn from columns (results not shown).

**Transphosphatidylation.** Phospholipase D activity was measured by detection of PtdBut formation (Rumsby *et al*, 2011) with TLC plates developed in CHCl<sub>3</sub>:MeOH: acetic acid (65:15:2, v/v) after Chahdi *et al*, (2003). Lipids were detected with iodine vapour and areas of adsorbent corresponding to PtdBut, phosphatidic acid (PtdOH) and PtdCho were scraped into vials with 2 ml scintillant to measure radioactivity. Means of triplicate d.p.m. values were calculated and PtdBut d.p.m. expressed as a % of PtdCho d.p.m. Cells in 6-well plates plus controls were treated with phorbol ester (TPA), 1 mM sodium oleate, 15 μM mastoparan 7 or the inactive form M17 for 30 min prior to extraction.

**Etn and Cho metabolite release in the presence of serum.** PNT2C2 cells (7.5 × 10<sup>4</sup>) in R2.5 in 24-well plates were labelled

with either 0.5  $\mu\text{Ci}$  [ $^3\text{H}$ ]-Etn or [methyl- $^3\text{H}$ ]-choline chloride (Perkin-Elmer) as above. Cells were rinsed and finally 0.5 ml R0, R10, R0/TPA or R10/TPA added to triplicate wells. Choline or Etn metabolite release was then followed up to 180 min as above.

**Sodium oleate and mastoparan.** Sodium oleate (Sigma-Aldrich, Poole, UK) was suspended in medium at 1 mM, warmed to 37 °C and dispersed by sonication. The pH was adjusted to 7.4 with 0.1 M HCl and the stock 1 mM solution diluted with serum-free medium as appropriate. Oleic acid (Sigma-Aldrich) was dissolved in DMSO and diluted in medium for use. Mastoparan 7 (Cambridge Bioscience, Cambridge, UK) and inactive Mastoparan 17 (Sigma-Aldrich) were dissolved in sterile water to 3 mM and then diluted to 5 and 15  $\mu\text{M}$  in medium. Cells were labelled with [ $^3\text{H}$ ]-Etn as above.

**Ionomycin.** Stock 10 mM ionomycin was diluted in DMSO to 1 mM and added to medium over cells to give final concentrations of 1  $\mu\text{M}$  and 2.5  $\mu\text{M}$ . Basal medium contained DMSO.

**Proliferation studies.** Effects of Etn and epidermal growth factor on LNCaP and PC3 cell proliferation were monitored after 48 h by MTS (Promega). Stimulation of DNA synthesis by Etn and EGF was also examined using the Click-iT EdU assay with Alexa Fluor azide as described by the manufacturer (Invitrogen, Paisley, UK).

**Effect of Etn on Cho uptake by PC3 cells.** PC3 cells ( $7.5 \times 10^4$ ) were cultured overnight in wells of 24-well plates in F7 medium. The F7 was then replaced with 0.5 ml Hanks balanced salt solution (HBSS) and cells incubated for 60 min at 37 °C. HBSS (0.5 ml) containing 0–50  $\mu\text{M}$  Etn was then added. After 5 min, 10  $\mu\text{l}$  of 250  $\mu\text{M}$  Cho containing 1  $\mu\text{Ci}$  [ $^3\text{H}$ ]-Cho was added to each well giving a final Cho concentration of 5  $\mu\text{M}$  (Lipton *et al*, 1988). Cells were incubated for 3 min (Lipton *et al*, 1988) prior to rinsing three times with cold (4 °C) HBSS. Cells were completely drained of rinse medium and solubilised in 200  $\mu\text{l}$  RIPA buffer. Radioactivity in triplicate 20  $\mu\text{l}$  aliquots was measured by scintillation counting.

**Statistics.** Statistical significance was determined by Student's two-tailed *t*-test as previously (Rumsby *et al*, 2011). The symbols \*, \*\* and \*\*\* denote statistically significant increases or decreases between unstimulated and stimulated cells or between stimulated and inhibited cells at  $P < 0.01$ ,  $P < 0.001$  and  $P < 0.0001$ , respectively.

## RESULTS

**Prostate epithelial cell lines do not incorporate [ $^3\text{H}$ ]-Etn into PtdCho.** Incubation of prostate epithelial cell lines with [ $^3\text{H}$ ]-Etn for 36 h followed by lipid extraction and TLC resolution of PtdEtn and PtdCho indicated that between 93 and 97% of the [ $^3\text{H}$ ]-label was associated with PtdEtn with only 3 and 6.7% being detected in PtdCho (Table 1). [ $^3\text{H}$ ]-label detected in Cho released into medium from [ $^3\text{H}$ ]-Etn-labelled PNT2C2, PNT1A and PC3 cells after 3-h experiments was also negligible compared with that in Etn (results not shown). Thus, any Cho metabolites released to the medium because of PtdCho turnover (Rumsby *et al*, 2011) will not interfere with quantitation of [ $^3\text{H}$ ]-Etn metabolites.

**Basal release of Etn metabolites is not influenced by cell density.** After 36 h of labelling with [ $^3\text{H}$ ]-Etn, PNT2C2 cells initially seeded at  $1 \times 10^5$  cells per well were fully confluent completely covering the well surface whereas cells seeded initially at  $2.5 \times 10^4$  cells were only 60–70% confluent. The basal ratio of GPEtn:PEtn:Etn metabolites released by non-confluent and confluent PNT2C2 cells was essentially the same at 3:1:6 (see PNT2C2 in Figure 4) indicating that the ratio of Etn metabolites released was not influenced by variation in initial cell density.

**Basal and TPA-stimulated Etn metabolite release.** Unstimulated cell lines all released [ $^3\text{H}$ ]-Etn metabolites to the medium in a typical 6-h time course (Figure 1), the longest experimental period. This basal [ $^3\text{H}$ ]-Etn metabolite release was linear with time. Phorbol ester stimulated [ $^3\text{H}$ ]-Etn metabolite release from PNT2C2 and PNT1A over the 6-h time course (Figure 1). TPA had no significant effect on Etn metabolite release from P4E6 and LNCaP cells for the first 3 h of the 6-h time course and, for LNCaP cells not even after 6 h. PC3 cells showed the most significant increase in [ $^3\text{H}$ ]-Etn metabolite release in response to TPA, stimulation being most marked over the first 3 h (Figure 1). These effects are reflected in the 3-h TPA stimulated/basal ratio of 1.5:1.3:1.09:0.94:2.2 for PNT2C2:PNT1A:P4E6:LNCaP:PC3 cell lines, respectively. In repeat experiments at 3 h, PC3 cells routinely showed the highest TPA:basal stimulation ratio (2.1–3.5-fold) compared with PNT2C2 (1.8–2.2) and PNT1A cells (1.5–2.4). P4E6 and LNCaP cell lines showed no reproducible increase in Etn metabolite release in response to TPA in repeat 3-h stimulations (Figure 1, Figure 2A and B). Phorbol ester-stimulated Etn metabolite release by PNT2C2, PNT1A and PC3 cell lines was inhibited by Ro31-8220, GF109203X (Figure 2A) and D609 (Figure 2B).

Table 1. Distribution of [ $^3\text{H}$ ]-label between PtdEtn and PtdCho after labelling prostate epithelial cell lines with [ $^3\text{H}$ ]-ethanolamine for 36 h

Cell line	PtdEtn	PtdCho	% [ $^3\text{H}$ ]	
	d.p.m. $\pm$ s.d.	d.p.m. $\pm$ s.d.	PtdEtn	PtdCho
PNT2C2	99666 $\pm$ 1196	3355 $\pm$ 332	96.7	3.3
PNT1A	124201 $\pm$ 10205	4726 $\pm$ 631	96.3	3.7
P4E6	23464 $\pm$ 4977	1569 $\pm$ 85	93.3	6.7
LNCaP	69166 $\pm$ 2095	3997 $\pm$ 495	94.5	5.5
PC3	16221 $\pm$ 7141	1060 $\pm$ 511	93.9	6.1

Abbreviations: d.p.m. = disintegrations per minute; PtdEtn = phosphatidylethanolamine; PtdCho = phosphatidylcholine; s.d. = standard deviation. Results are means values of triplicate TLC separations as described in Methods and are  $\pm$  s.d. ( $n = 3$ ).

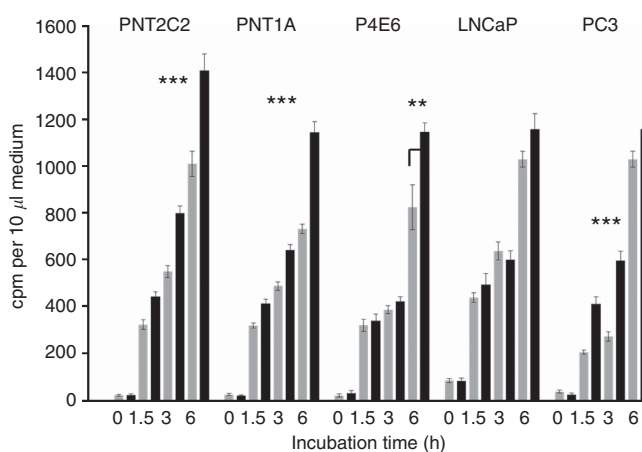
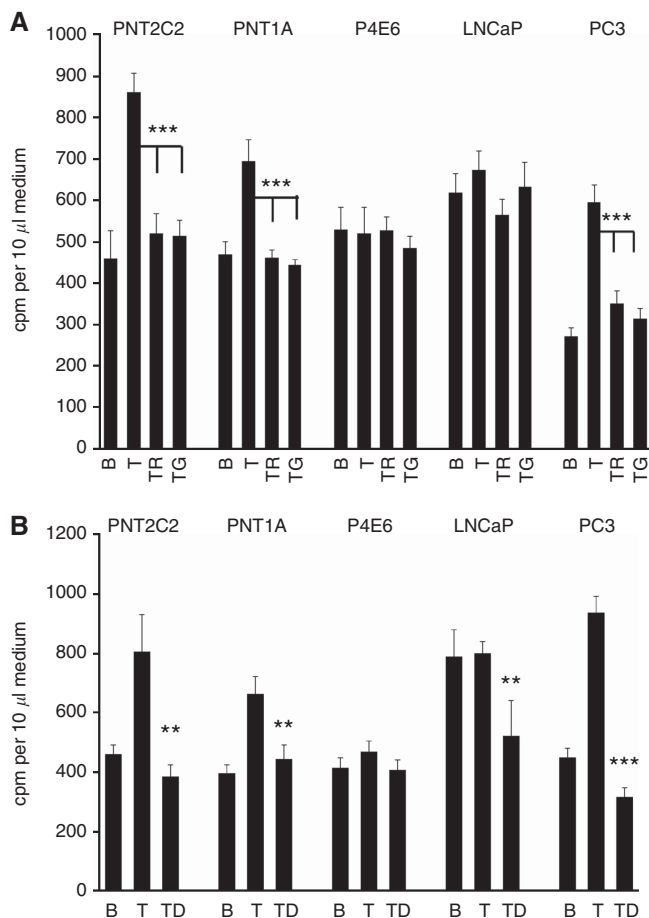


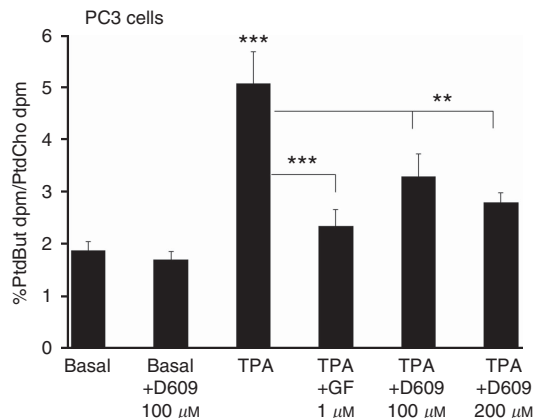
Figure 1. Effect of phorbol ester on Etn metabolite release by benign and cancer-derived human prostate epithelial cell lines. Cells were labelled with [ $^3\text{H}$ ]-Etn as described in Methods. After rinsing, cells were incubated with serum-free basal medium (□) or with basal medium containing 1  $\mu\text{M}$  TPA (■). [ $^3\text{H}$ ]-Etn metabolite release to the medium was measured at time zero and after 1.5, 3 and 6 h. Results are means  $\pm$  s.d. ( $n = 6$ ) and are typical of repeats. (\*\*\*)  $P < 0.0001$ , \*\*  $P < 0.001$  for phorbol ester stimulation against basal.



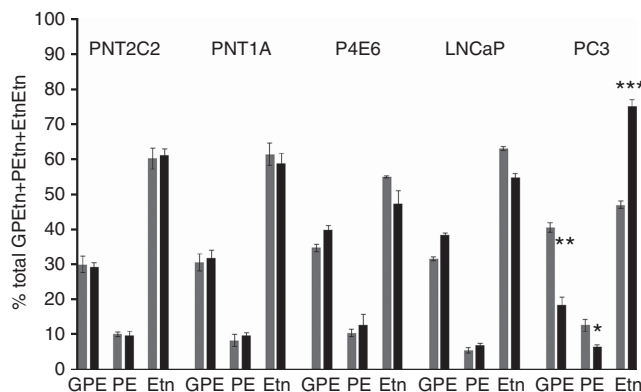
**Figure 2. (A).** Ro31-8220 and GF109203X inhibit phorbol ester-stimulated release of Etn metabolites from human prostate epithelial cell lines. Cells were cultured and labelled with [ $^3$ H]-Etn as described (Methods). After rinsing, cells were incubated for 3 h with appropriate serum-free basal (B) medium (+DMSO vehicle) or basal medium containing 1  $\mu$ M TPA (T), TPA + 1  $\mu$ M Ro31-8220 (TR) or TPA + 1  $\mu$ M GF109203X (TG). Results are means  $\pm$  s.d. ( $n=6$ ) and are typical of repeats. \*\*\*  $P<0.0001$  for inhibitor effects against TPA stimulated release. **(B).** Effect of D609 on TPA-stimulated release of [ $^3$ H]-labelled Etn metabolites by prostate epithelial cell lines. Cells were seeded, labelled and rinsed as described (Methods). After rinsing, cells were incubated for 3 h with appropriate serum-free basal (B) medium (+DMSO vehicle) or basal medium containing 1  $\mu$ M TPA (T) or TPA + 100  $\mu$ M D609 (TD). Results are mean values  $\pm$  s.d. ( $n=6$ ). \*\*\* $P<0.0001$  and \*\* $P<0.001$  for inhibitor effects against TPA stimulated release.

**D609 reduces TPA-stimulated PLD activity in a transphosphatidyl reaction.** D609 at 100  $\mu$ M and 200  $\mu$ M significantly inhibited TPA-stimulated PtdBut formation by PC3 cells in the transphosphatidyl reaction indicative of an effect on PLD (Morris *et al.*, 1997). TPA-stimulated PtdBut formation was also inhibited by 1  $\mu$ M GF109203X (Figure 3). D609 at 100  $\mu$ M had no inhibitory effect on basal PtdBut formation.

**Etn metabolites released by cell lines.** Unstimulated cell lines released [ $^3$ H]-Etn metabolites to the medium in the order Etn > GPEtn > PEtn (Figure 4). This order was unchanged when PNT2C2 and PNT1A were stimulated with TPA (Figure 4) even though total [ $^3$ H]-Etn metabolite release was increased (Figure 1). In contrast, TPA treatment of PC3 cells both increased total [ $^3$ H]-Etn metabolite (Figure 1) and Etn release at the expense of GPEtn and PEtn (Figure 4).



**Figure 3.** D609 inhibits phorbol ester-stimulated PtdBut formation by PC3 cells in the transphosphatidyl reaction. PC3 cells were labelled for 6 h with [ $^3$ H]-myristate, rinsed in serum-free medium and stimulated with TPA, TPA + 1  $\mu$ M GF109203X (TPA + GF) or TPA + D609 at 100  $\mu$ M and 200  $\mu$ M in the presence of 0.3% n-butanol. After 60 min, cells were rinsed, extracted and PtdBut, PtdOH and PtdCho resolved as described in Methods. Results are % PtdBut d.p.m. / PtdCho d.p.m. and are mean values  $\pm$  s.d. ( $n=3$ ). For PtdBut formation, TPA against basal and GF against TPA \*\*\* $P<0.0001$ ; for D609 against TPA \*\* $P<0.001$ .

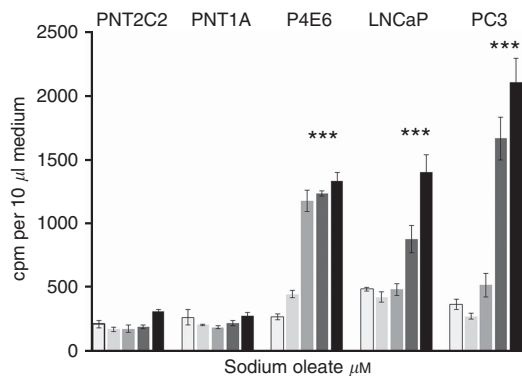


**Figure 4.** Metastatic PC3 cells upregulate Etn release on phorbol ester treatment. Cells were cultured and labelled with [ $^3$ H]-Etn (Methods). After rinsing, cells were incubated for 3 h with serum-free basal (□) medium (+DMSO) or basal medium containing 1  $\mu$ M TPA (■). Media were recovered and GPEtn, PEtn and Etn resolved by ion-exchange chromatography (Methods). Total d.p.m. associated with each fraction were calculated and results for each metabolite expressed as % of total GPEtn + PEtn + Etn d.p.m. Results shown are mean values  $\pm$  s.d. ( $n=3$ ) from a typical experiment. For PC3 cells, GPE, basal against TPA \*\* $P=0.005$ ; PE, basal against TPA, \* $P=0.04$ ; Etn, basal against TPA \*\*\* $P=0.0003$ .

**Phorbol ester stimulates Etn metabolite release in the presence of serum.** PNT2C2 cells were used to determine whether activation of PKC by TPA enhanced Etn metabolite release from cells already stimulated by serum. R10 medium (10% serum) increased basal Etn metabolite release by 1.3% (537  $\pm$  27 to 688  $\pm$  42 c.p.m./10  $\mu$ l medium) over a 3-h incubation. Addition of 1  $\mu$ M TPA to R10 medium boosted Etn metabolite release by 2.8% (537  $\pm$  27 to 1484  $\pm$  61 c.p.m./10  $\mu$ l medium).

**Oleate stimulates Etn metabolite release.** Oleate (1 mM) activates PLD2 in cultured mast cells (Sarri *et al.*, 2003). Therefore, this agent was used to examine whether PLD2 was involved in PtdEtn turnover (Figure 5). In initial 60-min experiments, PNT2C2 and





**Figure 5.** Etn metabolite release from prostate epithelial cell lines in response to varying NaOL concentrations. Cells were labelled with [ $^3\text{H}$ ]-Etn as described in Methods. After rinsing, cells were incubated at 37 °C in fresh medium containing 0 ( $\square$ ), 250 ( $\square$ ), 500 ( $\square$ ), 750 ( $\square$ ) or 1 mM NaOL ( $\blacksquare$ ) prepared as described. After an hour, 10  $\mu\text{l}$  aliquots of medium were removed for scintillation counting (Methods). Results are means  $\pm$  s.d. ( $n=6$ ). \*\*\* $P<0.0001$  for NaOL effects against basal.

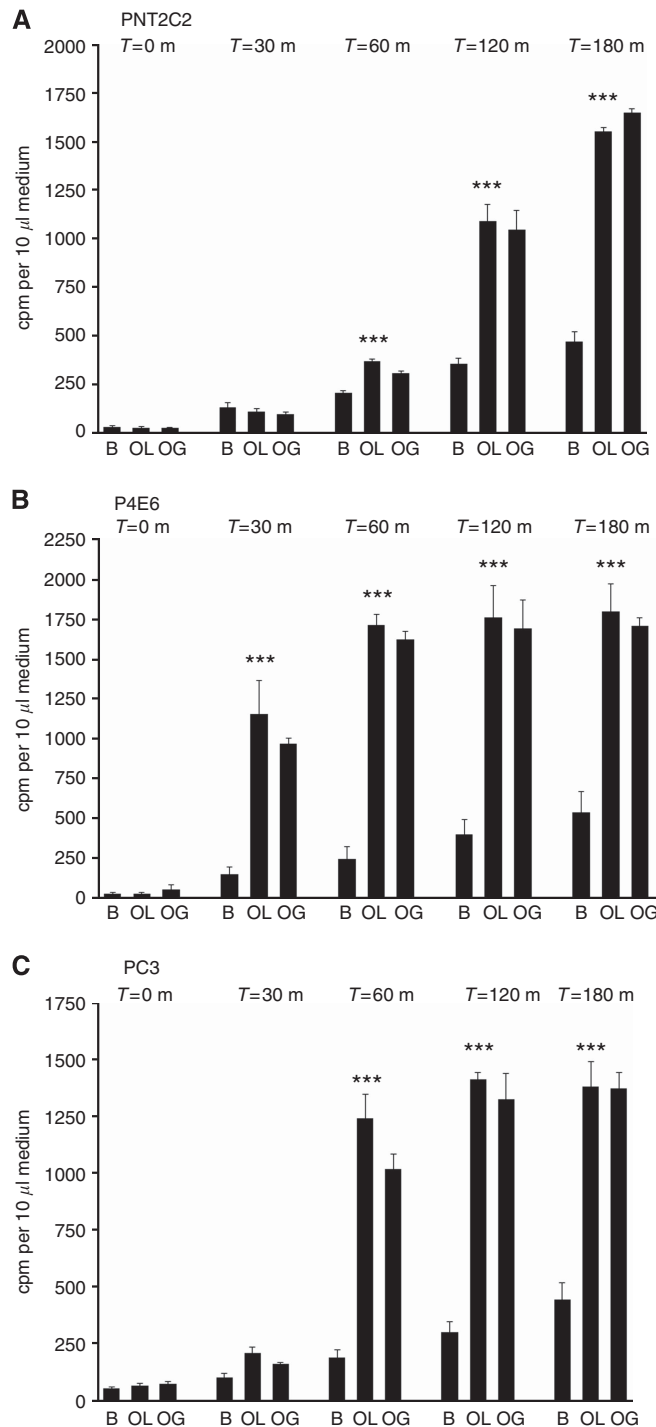
PNT1A cells were relatively unresponsive to oleate compared with tumorigenic cell lines. P4E6 cells especially showed a rapid release of Etn metabolites in response to oleate even at 500  $\mu\text{M}$  (Figure 5). In 3-h time course experiments, 1 mM oleate stimulated Etn metabolite release from PNT2C2, P4E6 and PC3 cell lines in a time-dependent manner (Figure 6A–C). Again, P4E6 cells responded more rapidly to oleate (Figure 6B) than the other cell lines. The oleate effect was not inhibited by 1  $\mu\text{M}$  GF109203X (Figure 6A–C) and only partially by 100  $\mu\text{M}$  D609 (results not shown). GlycerolphosphorylEtn and PEtn were the main metabolites released from the three cell lines after oleate stimulation, in all cases at the expense of Etn (Figure 7A–C).

**Mastoparan stimulates Etn metabolite release.** The cationic tetradecapeptide mastoparan selectively activates PLD2 in intact cells (Chahdi *et al*, 2003) and at 5  $\mu\text{M}$  and 15  $\mu\text{M}$  concentrations stimulated [ $^3\text{H}$ ]-Etn metabolite release from PNT2C2, P4E6 and PC3 cell lines (Figure 8A–C). GlycerolphosphorylEtn and PEtn were the main metabolites released from tumorigenic cell lines P4E6 and PC3 at the expense of Etn. Treatment of benign-derived PNT2C2 cells with mastoparan upregulated total Etn metabolite release (Figure 8A) but Etn metabolites were released in the same ratio as from unstimulated cells.

**Mastoparan but not sodium oleate stimulates PtdBut formation.** Incubation of PNT2C2 cells with 1 mM NaOL and n-butanol for 30 min did not stimulate PtdBut formation in the transphosphatidylation reaction. In a typical experiment basal PtdBut formation expressed as % d.p.m. PtdBut/d.p.m. PtdCho was 3.22%  $\pm$  1.01 (mean  $\pm$  s.d.,  $n=6$ ) whereas with sodium oleate stimulation the mean figure was 2.50  $\pm$  0.28 (s.d.,  $n=5$ ). For TPA-stimulated cells the PtdBut figure was 5.77%  $\pm$  0.55 over basal (see Figure 3). In duplicate experiments, mastoparan-7 stimulated PtdBut formation 1.33/1.7 times over basal in PNT2C2 cells, 2.8/2.6 times in P4E6 cells and 4.7/3.6 times in PC3 cells. Inactive M17 had no effect on PtdBut formation in any cell line.

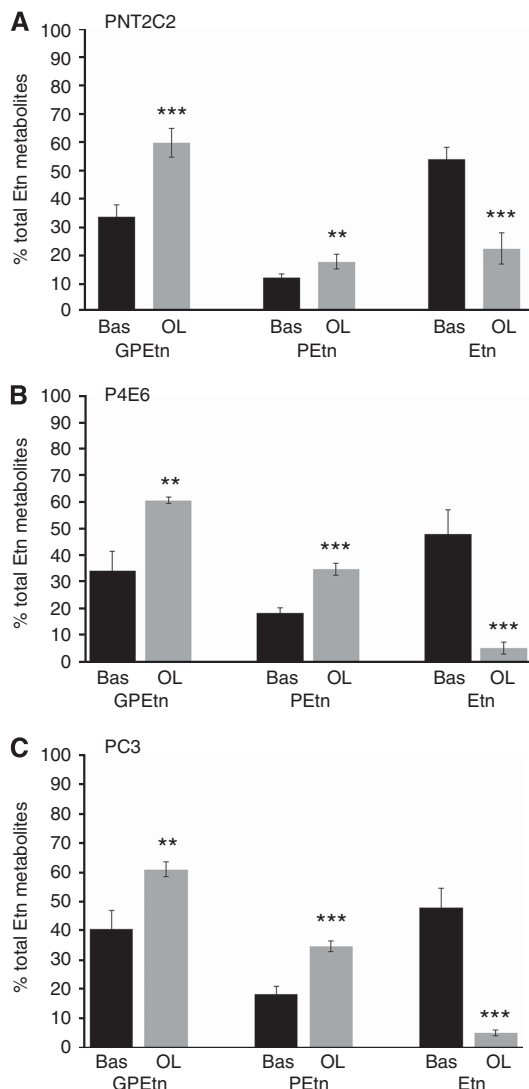
**Ionomycin stimulates GPEtn release.** Ionomycin at 2.5  $\mu\text{M}$  but not at 1  $\mu\text{M}$  stimulated a rapid release of Etn metabolites from PNT2C2 cells relative to basal (Figure 9A), an effect not observed with the PC3 cell line (Figure 9B). Ionomycin increased GPEtn release from PNT2C2 cells at the expense of Etn (Figure 9C) but had little effect on the ratio of Etn metabolites released from PC3 cells (Figure 9D).

**Etn does not stimulate LNCaP or PC3 cell proliferation but inhibits Cho uptake.** Etn at 20, 50 and 100  $\mu\text{M}$  did not stimulate



**Figure 6.** (A–C). Effect of 1 mM sodium oleate (OL) and oleate + 1  $\mu\text{M}$  GF109203X (OG) on Etn metabolite release from PNT2C2, P4E6 and PC3 cell lines. Cells were labelled with [ $^3\text{H}$ ]-Etn as described in Methods. After rinsing, cells were incubated at 37 °C in fresh medium containing 1 mM NaOL. Aliquots of medium were removed for scintillation counting (Methods) at the times shown. Results are mean  $\pm$  s.d. ( $n=6$ ) for a typical experiment from two repeats. For (A–C), \*\*\* $P<0.0001$  for NaOL effects against basal.

LNCaP or PC3 cell proliferation in a 48-h MTS assay (Figure 10A). In the same system, EGF at 20 and 50  $\text{ng ml}^{-1}$  stimulated LNCaP cell proliferation but not that of PC3 cells, which are unresponsive to this growth factor (El Sheikh *et al*, 2004). However, Etn did not synergise with EGF to further enhance LNCaP cell proliferation. In the Click-iT EdU assay, treatment of LNCaP cells with R10



**Figure 7. (A–C).** Oleate decreases Etn release from PNT2C2, P4E6 and PC3 cell lines and increases GPEtn and PEtn release. Etn metabolites in media from 3-h time course experiments were resolved by ion-exchange chromatography as described in Methods. Total d.p.m. for each metabolite was calculated and individual metabolites expressed as a % of GPEtn + PEtn + Etn. Results are mean values  $\pm$  s.d. ( $n = 6$ ) from duplicate media analyses. OL against basal values, (A),  $***P < 0.0001$ ,  $**P < 0.001$ ; (B),  $**P = 0.0002$ ,  $***P < 0.0001$ ; (C),  $**P = 0.006$ ,  $***P < 0.0001$ .

medium and PC3 cells with F7 medium for 48 h increased the % of green nuclei relative to total nuclei from  $3.8 \pm 1.98$  to  $20.97 \pm 1.99$  and  $3.8 \pm 1.198$  to  $23.7 \pm 2.8$ , respectively (results  $\pm$  s.d.,  $n = 3$ ). Etn at 10, 20 and  $50 \mu\text{M}$  had no significant proliferative effect on either LNCaP or PC3 cells. EGF at  $10 \text{ ng ml}^{-1}$  increased the % of green relative to total nuclei from  $3.8 \pm 1.64\%$  to  $10.1 \pm 3.0\%$  (results  $\pm$  s.d.,  $n = 3$ ) in LNCaP cells but had no effect on PC3 cells, in agreement with the MTS results above. Etn at a concentration of  $2.5 \mu\text{M}$  and higher in culture medium, which is normally Etn-free, reduces  $[^3\text{H}]\text{-Cho}$  uptake into PC3 cells by about 20% (Figure 10B).

## DISCUSSION

**Basal Etn metabolite release.** The time-dependent release of  $[^3\text{H}]\text{-Etn}$  metabolites to the medium (Figure 1) by all unstimulated

cell lines reflects the normal basal turnover of EtnPGs concentrated in the cytosolic leaflet of cell membranes (e.g., Vance 2008). None of the cell lines significantly methylated PtdEtn to PtdCho (Table 1) in keeping with findings that PtdEtn methylation pathways are mainly active in hepatocytes (Vance *et al*, 2007; Vance and Ridgway 1988). Hence, we can be confident that  $[^3\text{H}]\text{-label}$  released to the medium by cells is derived from turnover of EtnPGs. Etn is the major metabolite released (Figure 4) by cell lines. Phospholipase D is not generally involved in the basal turnover of phospholipids (Hii *et al*, 1991). However, in unstimulated LNCaP and PC3 cells, some basal PLD activity could contribute to Etn release because D609 reduced TPA-stimulated Etn metabolite release to below basal values (Figure 2B). D609 inhibits PLD in PC3 cells because we show that it blocks the PLD-specific transphosphatidyl reaction (Figure 3) in agreement with its effect on PLD in fibroblasts (Kiss and Tomono, 1995). D609 may inhibit PtdCho-PLC, PLA<sub>2</sub> and sphingomyelin synthase in other cell systems (Muller-Decker, 1989; van Dijk *et al*, 1997; Luberto and Hannun, 1998; Kang *et al*, 2008), but none of these enzymes release Etn directly from EtnPGs. Basal EtnPG turnover in the cell lines is not linked to PKC because neither Ro31-8220 nor GF109203X inhibit TPA-stimulated Etn metabolite release to below basal levels (Figure 2A). Further, GF109203X does not reduce TPA-stimulated PLD activity to below basal values in the transphosphatidyl reaction (Figure 3). Ro31-8220 and GF109203X are widely used PKC inhibitors (Gordge and Ryves 1994). Both inhibit several kinases (Alessi, 1997), but of these, only PKC is linked to activation of PLD1, and possibly PLD2 (Chen and Exton, 2004).

Basal Etn release could involve an N-acylPtdEtn-PLD (NAPE-PLD), N-acylethanolamide (NAE), N-acylEtn-hydrolysing acid amidase (NAAA) and/or fatty acid amide hydrolase (FAAH) pathway (Ueda *et al*, 2010; Coulon *et al*, 2012). N-acylPtdEtn is a minor prostate cell phospholipid, but its levels and that of NAE are elevated in prostate tumours (Schmid *et al*, 2002). mRNA and protein for NAAA, NAPE-PLD and FAAH are variously detected in normal and transformed prostate epithelial cells and cell lines and PCa tissue (Endsley *et al*, 2008; Wang *et al*, 2008). This pathway is relevant to PCa because NAEs generated by NAPE-PLD, especially anandamide (20:4 NAE), are endogenous cannabinoid receptor agonists and levels of cannabinoid receptors are elevated in PCa cells (Sarfaraz *et al*, 2005). Stimulation of cannabinoid receptors in LNCaP cells inhibits cell growth (Sarfaraz *et al*, 2005) and induces apoptosis (Sarfaraz *et al*, 2006). The antiproliferative effects of n-3 polyunsaturated fatty acids on cancer cells (Berquin *et al*, 2008) may be partly due to their conversion to NAE derivatives (Balvers *et al*, 2010; Brown *et al*, 2011) which then act on the cannabinoid receptors (de Petrocellis *et al*, 1998; Mimeault *et al* 2003; Brown *et al*, 2010).

Basal release of GPEtn by all cell lines suggests the involvement of a PLA<sub>2</sub> with lysophospholipase activity such as cPLA<sub>2</sub> $\alpha$  (Ghosh *et al*, 2006), which is active in PC3 cells (Patel *et al*, 2008). PhosphorylEtn is the minor Etn metabolite released by all cell lines on basal EtnGP turnover perhaps because high intracellular PEtn levels are maintained for the rate-limiting CTP:phosphoEtn cytidyltransferase reaction in EtnGP biosynthesis (Vance 2008). This would agree with spectroscopic results that PEtn is the major metabolite detected in intact benign and malignant prostate tissue (Swanson *et al*, 2008; Komoroski *et al*, 2011).

**Phorbol ester-stimulated Etn metabolite release.** Only the PC3 cell line used as a model of androgen-independent refractory stage PCa increased Etn release at the expense of GPEtn and PEtn on TPA treatment; this effect was not observed in LNCaP cells used to model androgen-sensitive disease. Taking the inhibitory effects of Ro31-8220, GF109203X and D609 into account, the most plausible explanation for this result is that a PKC-PLD1 pathway hydrolysing

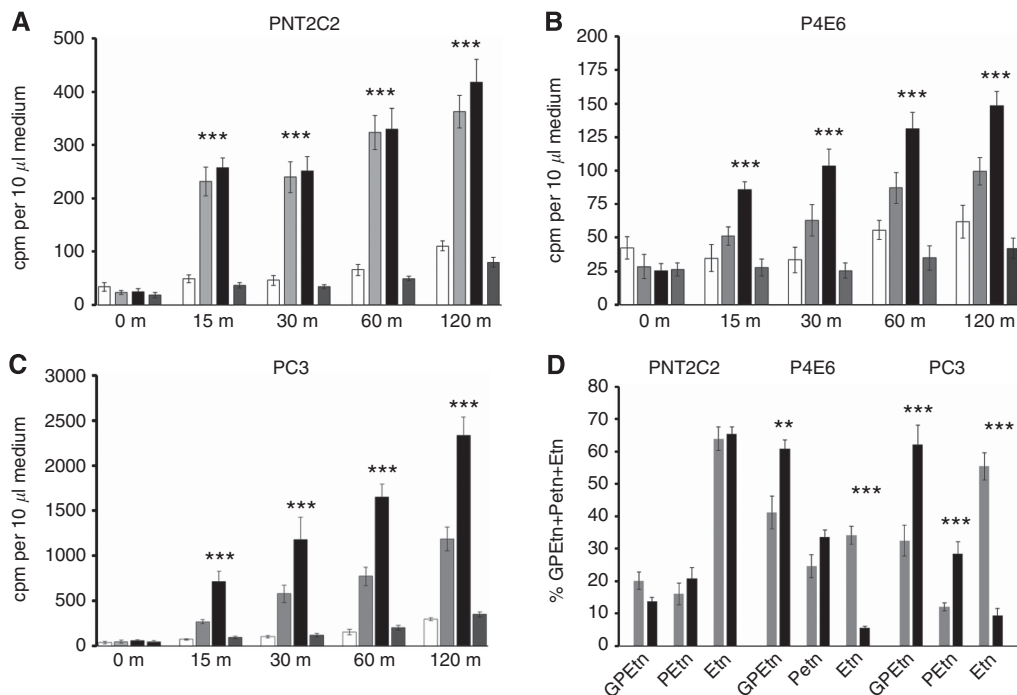


Figure 8. (A–C). Mastoparan-7 stimulates Etn metabolite release from PNT2C2, P4E6 and PC3 cell lines, and D, increases GPEtn and PEtn release from P4E6 and PC3 cell lines at the expense of Etn. Cells were labelled with (1-<sup>3</sup>H)-Etn as described in Methods. After rinsing, cells were incubated at 37 °C in fresh medium containing 0 (□), 5 (▒) and 15 μM (■) mastoparan-7 or 15 μM inactive mastoparan 17 (▤). Aliquots of medium were removed at the time intervals shown for scintillation counting (Methods). In (D), Etn metabolites were resolved by ion-exchange chromatography (Methods). In (A–C), Mastoparan 7 (15 μM) against basal, \*\*\**P*<0.0001. In (D), mastoparan-7 against basal, \*\**P*<0.001, \*\*\**P*<0.0001.

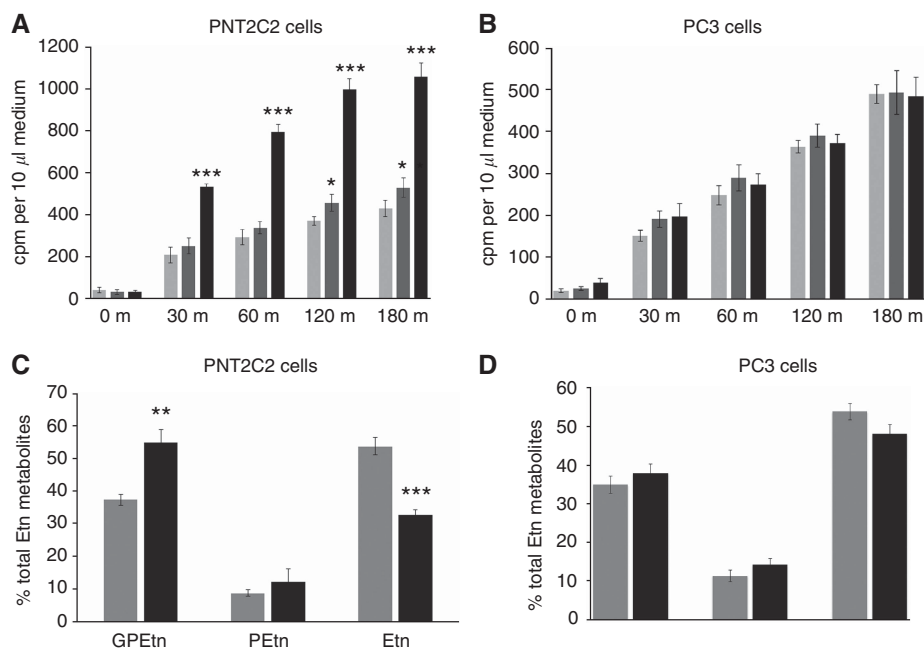
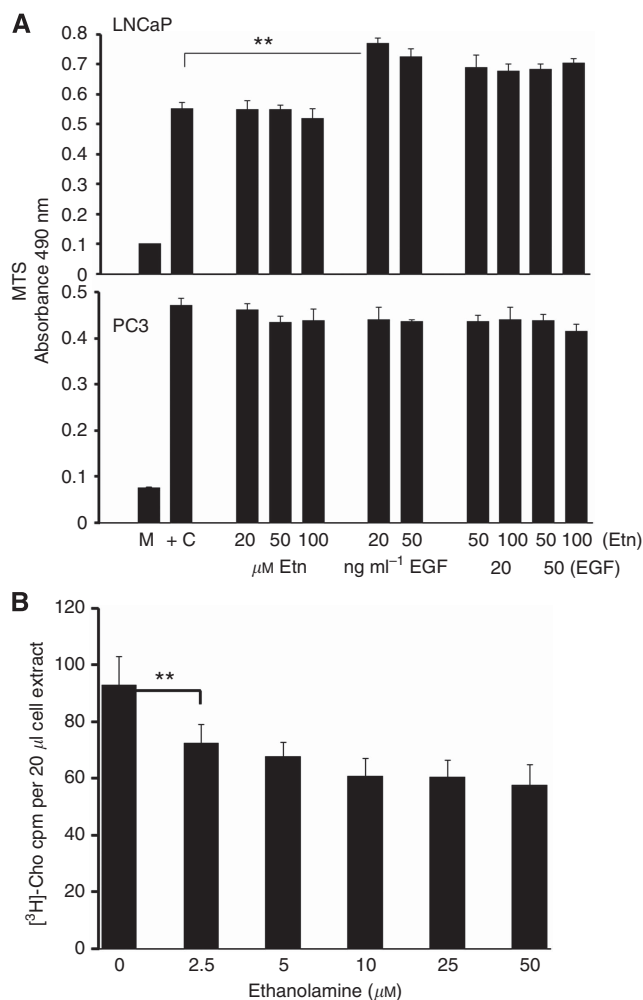


Figure 9. (A, B). Ionomycin promotes Etn metabolite release from PNT2C2 (A) but not from PC3 (B) prostate cell lines. (C, D). Ionomycin stimulates GPEtn release from the benign PNT2C2 but not from the metastatic PC3 cell line. Cells were labelled with [1-<sup>3</sup>H]-Etn as described in Methods. After rinsing, cells were incubated at 37 °C in basal medium containing DMSO vehicle (□) or 1 (▒) or 2.5 μM (■) ionomycin. Aliquots of medium were removed at the time intervals shown for scintillation counting. Results are mean d.p.m./10 μl medium and are +/– s.d. (*n* = 6). In (C and D), Etn metabolites in media from basal- (▒) and ionomycin- (■) treated cells were resolved by ion-exchange chromatography (Methods). Results are presented as in the legend for Figure 4. In A, ionomycin against basal \*\*\**P*<0.0001. In (C), ionomycin against basal \*\**P*<0.001, \*\*\**P*<0.0001.

EtnPGs is activated in this cell line and not in the other cell lines examined (McDermott *et al*, 2004). Basally active PLD2 may also be involved, but its regulation by PKC is not fully resolved (e.g., Han

*et al*, 2002; Chen and Exton 2004; Gomez-Cambronero 2011). Phosphatidylcholine is usually regarded as the main substrate for PLD1 (Pettitt *et al*, 2001; Jenkins and Frohman 2005), but our



**Figure 10. (A)** Etn does not stimulate LNCaP or PC3 cell proliferation. Cells in serum-free medium were treated as shown with Etn, EGF or EGF + Etn for 48 h and cell proliferation measured in an MTS assay. Results are mean MTS absorbance values  $\pm$  s.d. ( $n=6$ ),  $**P<0.001$  for EGF against basal. **(B)** Etn reduces [ $^3$ H]-Cho uptake into PC3 cells. PC3 cells were incubated in Hank's balanced salt solution containing different concentrations of Etn as described in Methods. Results shown are Etn effects on [ $^3$ H]-Cho uptake after 3 min and are the mean of replicates  $\pm$  s.d. ( $n=3$ ).  $**P<0.001$  for 2.5  $\mu$ M Etn against zero Etn.

findings indicate that PtdEtn and/or PlasEtn species are additional PLD1 substrates in PC3 cells on PKC stimulation as in other mammalian cells (Singh *et al*, 2005; Kiss *et al* 1994; Kiss and Tomono 1995). However, in PC3 cells, PtdEtn/PlasEtn is not an exclusive PLD1 substrate as observed in MCF-7/MDR cells (Kiss *et al*, 1994) because Cho release also increases on TPA treatment (Rumsby *et al*, 2011). Phorbol ester increases total Etn metabolite release from benign PNT2C2 and PNT1A cells (Figure 1) as for Cho metabolites (Rumsby *et al*, 2011), but this appears to occur by an upregulation of basal mechanisms of EtnGP turnover because the GPEtn:PEtn:Etn ratio does not change (Figure 4).

**Involvement of PLD2.** Oleate at low mM concentrations activates PLD2 in intact mast cells (Sarri *et al*, 2003); hence, we tested whether it would stimulate EtnPG turnover in the PCa cell lines. Oleate certainly stimulated total Etn metabolite release, but this was not due to activation of PLD2 because release of Etn decreased relative to GPEtn and PEtn, and further, oleate did not stimulate PtdBut formation in the PLD-specific transphosphatidylation reaction. This result was surprising for PC3 cells, which express

both PLD1 and PLD2 (Gibbs and Meier 2000). The increased formation of GPEtn by oleate suggests that a PLA<sub>2</sub> with lysoPA activity such as cPLA<sub>2</sub> $\alpha$ , which requires Ca<sup>2+</sup>, could have been activated (Ghosh *et al*, 2006). A rise in intracellular Ca<sup>2+</sup> could be triggered by oleate action on L-type Ca<sup>2+</sup> channels (e.g., Chang *et al*, 2000; Tian *et al*, 2009) expressed by LNCaP and PC3 cell lines (e.g., Huang *et al*, 2005; Sun *et al*, 2006; Gackiere *et al*, 2008) and/or by oleate activation of the fatty acid receptor GPR40 expressed in PCa cell lines (Rumsby, unpublished), which is linked to a rise in intracellular Ca<sup>2+</sup> (Hardy *et al*, 2005). Ionomycin increases GPEtn release at the expense of Etn from PNT2C2 cells supporting the idea that the observed oleate effect involves a rise in intracellular Ca<sup>2+</sup>. This was not observed with PC3 cells (Figure 9B and D) highlighting another difference between these prostate epithelial cell lines. Oleate treatment caused reversible cell rounding, which might also have allowed Ca<sup>2+</sup> entry and cPLA<sub>2</sub> activation. These results indicate that oleate does not activate PLD2 in prostate cell lines unlike its effects in mast cells (Sarri *et al*, 2003). The cationic tetradecapeptide mastoparan reportedly activates PLD2 in intact mast cells (Chahdi *et al*, 2003) and we found that it stimulated PtdBut formation in the PLD-specific transphosphatidylation reaction. However, it did not specifically stimulate PLD in P4E6 and PC3 cell lines because GPEtn and PEtn were the main metabolites released suggesting a preferential activation of PLA<sub>2</sub> and PtdEtn-PLC as observed in other cell types (Argiolas and Pisano, 1983; Schnabel *et al*, 1997; Ferry *et al*, 2001). With benign PNT2C2 cells, mastoparan upregulated basal mechanisms of EtnGP turnover because Etn metabolites were released in the same ratio as from unstimulated cells.

**Relevance to PCa.** Upregulated protein expression of PKC $\alpha$  and increased signalling by growth factors such as EGF that activate PKC are features of PCa (Cornford *et al*, 1999; de Miguel *et al*, 1999; Koren *et al*, 2004; Lahn *et al*, 2004; Stewart and O'Brian, 2005). Such observations suggest that a PKC-PLD1 pathway may be upregulated in PCa cells as is observed for PLD in other cancer cells and transformed cell lines (Foster and Xu, 2003). Androgen-dependent LNCaP and androgen-independent PC3 cell lines are widely used models of early and later stages of PCa, respectively, and our results suggest that these two cell lines differ in the regulation of PtdCho and EtnPG turnover by PKC as judged by the release of Cho and Etn metabolites. Androgen-independent PC3 cells appear to possess a PLD1 pathway that is upregulated on activation of PKC leading to increased turnover of both EtnPGs and PtdCho (Rumsby *et al*, 2011). This pathway and/or the ability to release Cho and Etn metabolites on PKC activation appears to be lacking in LNCaP cells. The extra PtdOH generated by the increased turnover of EtnPG in PC3 cells would maintain a cancer phenotype through mTOR (Foster, 2009; Toschi *et al*, 2009) in addition to that from PtdCho turnover by PLD1. It would also increase signalling through PtdOH (Wang *et al*, 2006) by promoting the membrane association of PtdOH-binding proteins (Stace and Ktistakis, 2006) including PI4P 5-kinase, which generates PI(4,5)P<sub>2</sub> essential for PLD activity (Jenkins and Frohman, 2005) and Raf-1 kinase and sphingosine kinase 1, both regulators of proliferation and apoptosis (e.g., Rizzo *et al*, 2000; Spiegel and Milstien 2002). Phosphatidic acid is also implicated in cell motility associated with metastasis (Mazie *et al*, 2006; Clarke *et al* 2009). Fatty acids in PtdOH derived from PtdEtn will be more unsaturated than those from PtdCho (Pettitt *et al* 1997; Weisser and Krieg 1998) while PtdOH from PlasEtn will contain 1-*O*-alkyl or 1-*O*-alkenyl chains. Such structural differences may result in EtnPG-derived PtdOH having different signalling, protein interaction, membrane fusion and fission properties, all linked to tumorigenesis (Jenkins and Frohman 2005; Wang *et al*, 2006). Phosphatidic acid is also readily converted by PLA<sub>2</sub> to lysoPtdOH,



an autocrine mediator in PCa cells (Daaka, 2002; Xie *et al*, 2002; Gibbs *et al*, 2009) promoting metastatic cell proliferation and motility.

Etn did not enhance PC3 cell growth as reported for other cell types (Kano-Sueoka *et al*, 1979; Murakami *et al*, 1982; Arthur and Lu, 1993; Kiss *et al*, 1997; Sasaki *et al*, 1997; Kume and Sasaki, 2006). However, at low concentrations, it did reduce Cho uptake as noted by others (e.g., Yorek *et al*, 1986; Lipton *et al*, 1988), hence it could modulate Cho uptake *in vivo*. Preferential uptake of Etn at the expense of Cho (Mintz *et al*, 2008) will increase EtnPG synthesis, regulating the membrane PtdCho:EtnPG ratio. This is tightly controlled because a deficiency of EtnPG causes abnormal PKC activity (Bazzi *et al*, 1992; Kano-Sueoka and Nicks, 1993) and impaired EGF binding to its receptor (Kano-Sueoka *et al*, 1990) as well as influencing the membrane association and function of PtdEtn-binding proteins, such as anti-apoptotic hPEBP4 (Li *et al*, 2007; Li *et al*, 2014) and Raf Kinase inhibitor protein, RKIP (Keller *et al*, 2005).

The use of spectroscopic methods to resolve phospholipid headgroup metabolites in the detection, diagnosis and characterisation of PCa (Kurhanewicz and Vigneron, 2008; DeFeo *et al*, 2011) and the suggestion that EtnPG metabolism may be a better marker for detection of PCa by spectroscopy than PtdCho (Komorowski *et al*, 2011) makes it important to understand how turnover of PtdCho and EtnPGs in PCa cells is regulated. Our results suggest that signalling pathways from PKC to PLD1 regulating turnover of PtdCho and EtnPGs differ in LNCaP and PC3 cell lines, two widely used models of early stage and later refractory stage PCa.

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