Journal of Cellular Biochemistry 103:1551–1572 (2008)

The Vitamin D Receptor-Mediated Activation of Phosphatidylinositol 3-Kinase (PI3Kα) Plays a Role in the 1α,25-Dihydroxyvitamin D_3-Stimulated Increase in Steroid Sulphatase Activity in Myeloid Leukaemic Cell Lines

Philip J. Hughes,[1]* Jimmy S. Lee,[2] Neil E. Reiner,[2] and Geoffrey Brown[1]

[1]Division of Immunity and Infection, The Medical School, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK
[2]Department of Medicine (Division of Infectious Diseases), University of British Columbia, Faculties of Medicine and Science, Vancouver Coastal Health Research Institute (VCHRI), Vancouver, British Columbia, Canada V5Z 3J5

Abstract In this article we show that 1α,25-dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$) stimulates the activity of the class IA phosphatidylinositol 3-kinase PI3Kα and its downstream target Akt in HL60, U937 and THP-1 myeloid leukaemic cell lines. Furthermore, we show that the classical nuclear vitamin D receptor (VDR_{nuc}) is involved in this activation of the PI3K/Akt signalling in these cell lines. We have previously shown that the activity of steroid sulphatase is stimulated in HL60, U937 and THP-1 myeloid leukaemic cell lines by 1α,25$(OH)_2D_3$ (Hughes et al., [2001] Biochem J 355:361–371; Hughes et al. [2005] J Cell Biochem 94:1175–1189; Hughes and Brown [2006] J Cell Biochem 98:590–617). In this article we show that the 1α,25$(OH)_2D_3$-stimulated increase in signalling via the PI3K/Akt pathway plays a role in the increase in steroid sulphatase activity in the HL60 U937 and THP-1 cell lines. We used a variety of pharmacological and biochemical approaches to show that activation of PI3Kα mediates the 1α,25$(OH)_2D_3$-stimulated increase in steroid sulphatase activity in myeloid leukaemic cells. We also show that the PI3K/Akt dependent activation of NF-κB plays a role in the 1α,25$(OH)_2D_3$-stimulated increase in steroid sulphatase activity in myeloid leukaemic cells. J. Cell. Biochem. 103: 1551–1572, 2008.

Key words: vitamin D receptor; phosphatidylinositol 3-kinase; genomic and non-genomic signalling; steroid sulphatase; myeloid cells

Recently, we have shown that the activity/expression of the microsomal membrane-bound enzyme steroid sulphatase (often called arylsulphatase C, EC 3.1.6.2) is increased by 1α,25-dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$) in several myeloid leukaemic cell lines [Hughes et al., 2005]. Steroid sulphatase converts the 'inactive' estrogen and androgen pre-cursors estrone sulphate (E_1S) and dehydroepiandrosterone sulphate (DHEAS) into 'active' steroids within cells [Reed et al., 2005].

It is generally assumed that 1α,25$(OH)_2D_3$ associates with its cognate nuclear receptor (VDR_{nuc}) in the nucleus to generate its biological effects. In this scenario, the liganded VDR_{nuc} dimerises with the retinoid receptor-X (RXR) in the nuclear cytoplasm. This heterodimer binds to vitamin D response elements (VDRE) in the promoter regions of 1α,25$(OH)_2D_3$-responsive genes to initiate an exchange of transcriptional repressors for transcriptional activators and drive the

Grant sponsor: Leukaemia Research Fund, United Kingdom; Grant number: LRF-0257; Grant sponsor: Canadian Institutes of Health Research; Grant number: MOP-8633.

*Correspondence to: Philip J. Hughes, Division of Immunity and Infection, The Medical School, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK. E-mail: P.J.Hughes@bham.ac.uk

Received 20 October 2006; Accepted 19 July 2007

DOI 10.1002/jcb.21545

transcription of responsive genes [Jones et al., 1998]. These 'direct genomic' responses are slow and may take hours or days to become apparent. Recent evidence suggests that the classical model of nuclear receptor activation cannot account for all the observed biological actions of $1\alpha,25(OH)_2D_3$. For example, there are a large number of $1\alpha,25(OH)_2D_3$-responsive genes that do not have a discernible VDRE in their promoter regions [Wang et al., 2006]. Furthermore, it has become increasingly accepted that, like other steroid hormones, some of $1\alpha,25(OH)_2D_3$ biological effects are mediated by the activation of rapid non-genomic signalling pathways [Norman, 2006]. These rapid 'non-genomic' responses to $1\alpha,25(OH)_2D_3$ occur within minutes of stimulation and are initiated at the level of the cell membrane. There is considerable debate whether a small pool of classical VDR_{nuc} in the cytoplasm which translocates to the plasma membrane upon ligation [Norman, 2006] or an alternate plasma membrane-associated $1\alpha,25(OH)_2D_3$-binding protein ($1,25D_3$-MARRS), recently identified as ERp57 [Khanal and Nemere, 2007], drive $1\alpha,25(OH)_2D_3$-stimulated rapid non-genomic signals.

Non-genomic signals appear to play an important role in the $1\alpha,25(OH)_2D_3$-stimulated increase in steroid sulphatase activity in myeloid leukaemic cells. We have shown that VDR_{nuc}-mediated activation of the Ras/RAF/ERK-MAP kinase pathway plays an important role in the $1\alpha,25(OH)_2D_3$-stimulated increase in steroid sulphatase activity in myeloid leukaemic cell lines [Hughes and Brown, 2006]. Recent evidence suggest that the phosphatidylinositol 3-kinase (PI3K) non-genomic signalling system is also activated by $1\alpha,25(OH)_2D_3$ in myeloid cells. $1\alpha,25(OH)_2D_3$-mediated stimulation of PI3K appears to be essential for the monocytic differentiation of both HL60 cells [Tse et al., 2007] and THP-1 [Lee et al., 2004] myeloid leukaemic cell lines and prevents apoptosis of HL60 cells [Zhang et al., 2006]. In these myeloid cell lines, the $1\alpha,25(OH)_2D_3$-stimulated PI3K activity has been associated with an augmentation in Akt activity [Tse et al., 2007] and an increase NF-κB DNA binding and gene transcription [Tse et al., 2007] We have recently demonstrated a role for the PI3K/Akt/NF-κB signalling pathway in the retinoid-stimulated increase in steroid sulphatase activity in myeloid cells [Hughes et al., 2005]. In this study, we have used a combination of pharmacological inhibitors and genetic interference approaches to show that $1\alpha,25(OH)_2D_3$, acting via VDR_{nuc}, stimulates PI3Kα activity in myeloid leukaemic cells and demonstrate that increased signalling via the PI3Kα/Akt/NF-κB signalling cascade is essential for the $1\alpha,25(OH)_2D_3$-stimulated increase in steroid sulphatase activity.

EXPERIMENTAL PROCEDURES

Chemicals

$1\alpha,25(OH)_2D_3$ was a gift from Dr. Lise Binderup (Leo Pharmaceutical Products, Ballerup, Denmark). The VDR_{nuc} antagonists ZK159222 and ZK168281 were gifts from Dr. Andreas Steinmeyer (Medicinal Chemistry III, Schering-Plough AB, Berlin, Germany). The non-genomic VDR agonist $1\alpha,25$-dihydlumisterol$_3$ and the antagonist $1\beta,25$-dihydroxyvitamin D_3 were a gift from Prof. Anthony Norman (Department of Biochemistry, University of California at Riverside, CA). All radiolabelled compounds were purchased from Perkin-Elmer Life Science Products-UK, Ltd (Cambridge, UK). The non-specific PI3K inhibitors wortmannin, LY294004, the PI3Kα specific inhibitor PX-866 and the PI3Kδ inhibitor caffeine, the mTOR inhibitor rapamycin, the IKK inhibitor thalidomide and the heterotrimeric G-protein inhibitor pertussis toxin were purchased from the Sigma Chemical Co. (Poole Dorset, UK). The PIKα specific inhibitor 3-[4-(4-morpholinyl)thioeno[3,2-d]pyrimidin-2-yl]-phenol (compound 15e), the PI3Kβ inhibitor TGX-221 and PI3Kγ inhibitor AS-252424 were purchased from Alexis Bichemicals. The Akt inhibitors 1-L-6-hydroxymethyl-*chiro*-inositol-2I-2-*O*-methyl-3-*O*-octadecyl-carbonate (Akt inhibitor I), SH-5 (Akt inhibitor II) and *Tat-Akt-in* Akt inhibitory 15-mer peptide (NH_2-AVTDHPDRLWWEKF-COOH, AKT inhibitor VII), the 'non-specific' tyrosine kinase inhibitor herbimycin A and the Src tyrosine kinase inhibitor PP1 were purchased from Calbiochem (Nottingham, England, UK). The p85 blocking peptide *N*-acetyl-Asp-Tyr(2-malonyl)-Val-Pro-Met-leu-NH_2 (PI3K-SH2-OMT) and the IKK inhibitor wedelolactone were purchased from BIOMOL International LP (Exeter, Devon, UK). Specific antibodies directed against epitopes in the SH2-containing N-termini of either p110α (clone B-9, catalogue number sc-1637), p100β (clone H-198,

catalogue number sc-7175) catalytic subunits of PI3K and the VDR_{nuc} antibody (sc-1008) were purchased from Insight Biotechnology (London, UK). The AKT Face™ Elisa kit and the Chariot™ protein transfection reagent were purchased from Active Motif (Rixensart, Belgium).

Cell Culture

HL60, U937 and THP-1 acute myeloid leukaemia cell lines were grown in RPMI 1640 medium supplemented with 10% foetal calf serum, penicillin (100 U/ml) and streptomycin (100 μg/ml) in 95% air/5% CO_2 at 37°C. Cells were seeded at 2.5×10^5 cells per ml as 10 ml cultures in 25 cm^2 flasks and differentiation was induced with 0.1–100 nM 1α,25$(OH)_2D_3$ for 3 days unless otherwise stated.

A subline of the promyelocyte cell line U937 (U937:Δp85-p18 cells) that contains a dominant negative truncated PI3K p85 subunit (Δp85) under the control of an IPTG/PMA/$ZnCl_2$-sensitive CMV driven promoter was obtained from Dr. Phillip Hawkins (Inositide Signalling Laboratory, Babraham Institute, Cambridge, UK). Stock cultures of U937:Δp85-p18 cells were established in antibiotic- and FBS-supplemented RPMI 1640 medium containing 0.6 mg/ml G418 and 0.1 mg/ml hygromycin B. To induce expression of Δp85, the stock cell cultures were treated with 20 mM IPTG, 1 nM PMA and 100 μM $ZnCl_2$ for 16 h prior to experimentation. These inducing agents were removed just before cells were stimulated. This cell line has previously been used to show that a tyrosine kinase-linked PI3Ks are involved in FcγR1-mediated endocytosis of immune complexes [Gillooly et al., 1999] and FcγR1-mediated activation of phospolipase Cγ1 and PKC [Melendez et al., 1999].

The role of PI3Kα in 1α,25$(OH)_2D_3$-stimulated increase in PI3K activity was also investigated in a THP-1 cell line (THP-1:HR-p110α3) in which expression of the p110α catalytic subunit of PI3K was silenced by RNA interference (RNAi). A short hairpin RNA targeting the p110α catalytic subunit of PI3K was delivered using a lentiviral-vector exactly as described in Lee et al. [2004]. Expression of p110α was abrogated whilst the levels of expression of p110β and p110δ, or the p85α regulatory subunit were unchanged [Lee et al., 2004]. A mock transfected cell line (THP-1:HR-mock) was used as a control. Cells were grown as described in Lee et al. [2004].

Transfection of THP-1 Cells With p110 Phosphatidylinositol 3-Kinase Catalytic Subunit-Specific Antibodies Using the Chariot™ Protein Delivery Reagent

THP-1 cells were loaded with antibodies directed against the p110α, p110β or p110δ catalytic subunits of PI3K using the Chariot™ protein transfection kit (Active Motif) essentially as described by the manufacturer. In brief, 5 μg of antibody and 1 μg of β-galactosidase were dissolved in 100 μl of 2 × sterile phosphate-buffered saline (PBS) and mixed with 6 μl of sonicated Chariot transfection reagent (suspended in 100 μl sterile H_2O). This mixture was incubated at room temperature for 30 min to form the transfection complex reagent. THP-1 cells (5×10^5) were washed twice with pre-warmed serum-free RPMI 1640 and the final cell pellet was mixed with the antibody/Chariot mixture and incubated at 37°C in a tissue culture incubator. After 30 min a further 400 μl of serum free RPMI 1640 were added to the cells to achieve the final transfection concentration. The THP-1 cells were incubated at 37°C for a further 5 h to allow internalisation of the antibody. Control cells were treated with the Chariot reagent alone (6 μg in 200 μl of sterile PBS). After antibody loading the cells were washed twice in RPMI 1640 containing 10% FBS and re-suspended in a volume of 1 ml and aliquoted into individual wells in a 24 well tissue culture plate. As a second control at this point 2 μg of the appropriate antibody was added to aliquots of THP-1 cells that had been 'mock' transfected with PBS. At the end of the incubation period a 100 μl aliquot of cells was removed, fixed and stained for β-galactosidase expression as described in the manufacturers instructions to assess transfection efficiency. The remainder of the cells were spilt into two portions. One aliquot was used for to determine the effect of 100 nM 1α,25$(OH)_2D_3$ on PI3K activity. The other aliquot was stimulated with 100 nM 1α,25$(OH)_2D_3$ for 24 h and steroid sulphatase activity was measured as described below. Using this antibody loading protocol THP-1 cells remained >90% viable for at least 96 h after transfection. However, significant toxicity was observed in both HL60 and U937 cells within 48 h of protein transfection, so the

effect of p110 antibody loading was not pursued in these cell lines.

Measurement of Steroid Sulphatase Activity in Cell Fractions

Cells were collected by centrifugation and re-suspended in 1 ml of hypotonic homogenisation buffer (50 mM HEPES, 0.1 mM EDTA, pH 7.4). After 30 min on ice, the cells were disrupted by repeated sonication and centrifuged at 100,000*g* at 4°C for 60 min. The pellet was re-suspended in 0.5 ml of the same buffer by sonication and its protein concentration was measured (Bio-Rad kit, Hemel Hempstead, Herts, UK). Steroid sulphatase activity was assayed at 37°C in 0.02 M Tris-HCl (pH 7.5) containing 20 μM E_1S (spiked with ~100,000 dpm of [^{3}H]-E_1S) in a final volume of 100 μl. Reactions were started with 30–80 μg protein and were quenched after 60 min with 900 μl of ice-cold 0.1 M sodium bicarbonate containing 5,000 dpm of [^{14}C]-E_1 to determine the recovery of the extraction process. Nine hundred and fifty microlitres of the resulting mixture were extracted with 3 ml of toluene and the organic and aqueous layers were separated. Eight millilitres of Emulsifier Safe scintillant (Canberra Packard, Pangborne, Berks, UK) were added to each fraction and radioactivity was determined in a Packard 2000CA TriCarb liquid scintillation counter. The results were corrected for carry-over of radiolabelled substrate into the organic layer determined in blank incubations without cell protein.

In Vivo Labelling of Polyphosphoinositides, Lipid Extraction, and Separation by Thin-Layer Chromatography

Myeloid leukaemic cells were cultured overnight in RPMI 1640 containing 0.5% FBS and then washed twice in phosphate-free RPMI/0.5% dialysed FBS and finally re-suspended in the same medium containing carrier-free [^{32}P]-orthophosphate (2.0 mCi/ml) and incubated at 37°C for 3 h. The cells were washed twice in phosphate-free RPMI 1640/0.5% FBS/antibiotics and re-suspended at a cell density of 2×10^7 cells per ml. The cells were incubated at 37°C for 30 min. In some experiments cells were treated during this incubation period with the vitamin D receptor antagonists ZK159222 (500 nM) and ZK168281 (500 nM), 1β,25-dihydroxyvitamin D_3 (500 nM), an antagonist of non-genomic vitamin D signalling, tyrosine kinase inhibitors genistein (100 μM) or PP1 (25 μM) or the G-protein inhibitors suramin (100 μM) or pertussis toxin (2 μg ml^{-1}). Cells were stimulated with 100 nM 1α,25$(OH)_2D_3$ for 5 min in a final reaction volume was 400 μl. Reactions were terminated by the addition of 1.5 ml ice cold chloroform/methanol (1:2 v/v) containing butylated hydroxytoluene (1 mg ml^{-1}) and 10 μl of a freshly prepared 1:1:1 mixture of PtdIns/PtdIns(4)*P*/PtdIns(4,5)P_2 in chloroform (1 mg ml^{-1} of each lipid) was added as carrier. Lipids were extracted by standard methods and ^{32}P-phosphoinositides were separated by tlc using oxalate-impregnated silica gel plates with chloroform/acetone/methanol/acetic acid/water (80:30:26:24:14 v/v) as the mobile phase. The position of the ^{32}P-labelled phosphoinositides was compared with migration of unlabeled standards [PtdIns(4)*P*, PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3]. The spot corresponding to ^{32}P-PtdIns(3,4,5)P_3 was scraped and counted by liquid scintillation spectrometry. Due to the inherent variability of degree of ^{32}P-labelling in each experiment all results are expressed as the fold change from unstimulated cells.

Measurement of In Vivo AKT Activity in Myeloid Cells

1α,25$(OH)_2D_3$-stimulated AKT activity was measured using the FACE^{TM} AKT chemiluminescent Elisa assay kit (Actif Motif Europe, Rixensart, Belgium). The assay measures both total AKT and phosphorylated AKT (Ser473 phosphorylation) levels using sensitive primary antibodies and an HRP-conjugated secondary antibody. Myeloid cells were harvested by centrifugation and re-suspended at a concentration of 10^6 cells per ml in pre-warmed serum free RPMI 1640. After a 2 h equilibration period, the cell suspension was stimulated with 100 nM 1α,25$(OH)_2D_3$ for up to 24 h. One hundred microlitres of the cell suspension was removed and was labelled with crystal violet (100 μl) for 30 min. The cells were lysed by sonication and absorbance was measured at 595 nM. This was done to ensure that equal numbers of cells were assayed and to normalise data from the AKT assay. A duplicate 100 μl sample was taken and centrifuged at 30,000*g* for 60 s at 4°C to terminate the reaction. The supernatant was aspirated and cells were immediately fixed with 100 μl of 8% (v/v) formaldehyde in PBS for 30 min at room temperature. The samples were then processed as per the manufacturers

recommendations except that each reaction was carried out in a 1.5 ml eppendorf tube which was centrifuged for 30 s at 30,000*g* between each washing or labelling step. The final supernatant was transferred to a polycarbonate LP4 tube and chemiluminescence was measured in a Berthold LB 953 luminometer.

Statistical Analysis

The statistical significance between groups of data were analysed by the Student's *t*-test or where appropriate by the Mann–Whitney rank sum or Kruskal–Wallis one-way analysis of variance tests using either the SPSS (version.13.0) or the SigmaStat^{TM} statistical software packages. Irrespective of the test used a *P*-value <0.05 was considered to be a significant difference. Dose response or inhibition curves were fitted to Sigmoidal dose-response curves using the 'pharmacology' module of the Sigmaplot^{TM} (version 8.0) graphical software package.

RESULTS

1α,25-Dihydroxyvitamin D_3 Stimulates Tyrosine Kinase-Activated, but not Heterotrimeric G-Protein-Coupled, Phosphatidylinositol 3-Kinase Isoforms in HL60, U937 and THP-1 Myeloid Leukaemic Cells Lines

Figure 1A shows that 100 nM 1α,25$(OH)_2D_3$ simulated a biphasic increase in PI3K activity (as measured by an increase in ^{32}P-PtdIns(3,4,5)P_3) in serum-deprived ^{32}P-labelled HL60, U937 and THP-1 myeloid leukaemic cell lines. An increase in ^{32}P-PtdIns(3,4,5)P_3 content became apparent in each cell line within 1 min of stimulation and peaked ~4- to 8-fold over basal within 5–10 min of stimulation. The level of ^{32}P-PtdIns(3,4,5)P_3 gradually began to wain reaching a nadir some 60–120 min after stimulation followed by a second smaller rise in ^{32}P-PtdIns(3,4,5)P_3 levels that was still apparent 24 h after 1α,25$(OH)_2D_3$ addition (Fig. 1A). The 1α,25$(OH)_2D_3$-stimulated accumulation of ^{32}P-PtdIns(3,4,5)P_3 was blocked in all three myeloid leukaemic cell lines by >70% by pre-treatment with wortmannin (100 nM) and LY294002 (10 μM; data not shown). This early rise in PI3K activity produced by 1α,25$(OH)_2D_3$-stimulation was not blocked by pre-treating the cells with cycloheximide, indicating that this effect of 1α,25$(OH)_2D_3$ is mediated by pre-existing proteins (data not shown).

The Nuclear Vitamin D Receptor Is Involved in the 1α,25-Dihydroxyvitamin D_3-Stimulation of Phosphatidylinositol 3-Kinase Activity

Figure 1B shows that both 1α,25$(OH)_2D_3$ (100 nM) and 1α,25-dihydroxylumisterol$_3$ (100 nM) stimulated PI3K activity in HL60 cells. The maximum response produced by 1α,25-dihydroxylumisterol$_3$ was only ~60 % of that produced by 1α,25$(OH)_2D_3$. 1β,25-Dihydroxyvitamin D_3 (1β,25$(OH)_2D_3$) is a specific antagonist of the activation of non-genomic signalling by 1α,25$(OH)_2D_3$. Figure 1B also shows that the 1α,25$(OH)_2D_3$- and the 1α,25-dihydroxylumisterol$_3$-stimulated increases in PI3K activity in HL60 cells were reduced by a five-fold excess of the non-genomic antagonist 1β,25$(OH)_2D_3$. The vitamin D analogues ZK159222 and ZK168281 bind to the VDR_{nuc} with high affinity but have very low agonist activity and antagonise VDR_{nuc}-mediated transactivation [Herdick et al., 2000; Ociai et al., 2005]. Figure 1B shows that the 1α,25$(OH)_2D_3$ and 1α,25-dihydroxylumisterol$_3$-stimulated increases in PI3K activity were blocked by a fivefold excesses of ZK159222 and ZK16821. Pre-treatment with the RXR antagonist VTP195393 or the PPARγ antagonist GW9662 failed to have any effect on the 1α,25$(OH)_2D_3$-stimulated increase in PI3K activity in HL60, cells (data not shown). Similar results were obtained using U937 and THP-1 cells (data not shown).

1α,25-Dihydroxy Vitamin D_3 Preferentially Activates PI3Kα in Myeloid Leukaemic Cells

Myeloid cells express multiple PI3K isoforms, each of which has a distinct role in cellular proliferation, differentiation and chemotaxis. The three members of the class IA PI3K subfamily are heterodimers comprising of a unique catalytic subunit (p110α, p110β or p110δ) and a tightly associated regulatory subunit (p85). Convention has it that the class IA isoforms are activated downstream of tyrosine kinase coupled receptors. Perhaps the most abundant PI3K isoform in leucocytes is the class IB family member PI3Kγ, which is activated by receptors coupled to heterotrimeric G-proteins [Hawkins et al., 2006]. Depending on the cell type studied, 1α,25$(OH)_2D_3$ signals via tyrosine kinase- [Boland et al., 2005] or heterotrimeric G-protein activated-signalling pathways [Le Mellay et al., 1999]. Therefore, in theory 1α,25$(OH)_2D_3$ could activate any of the class

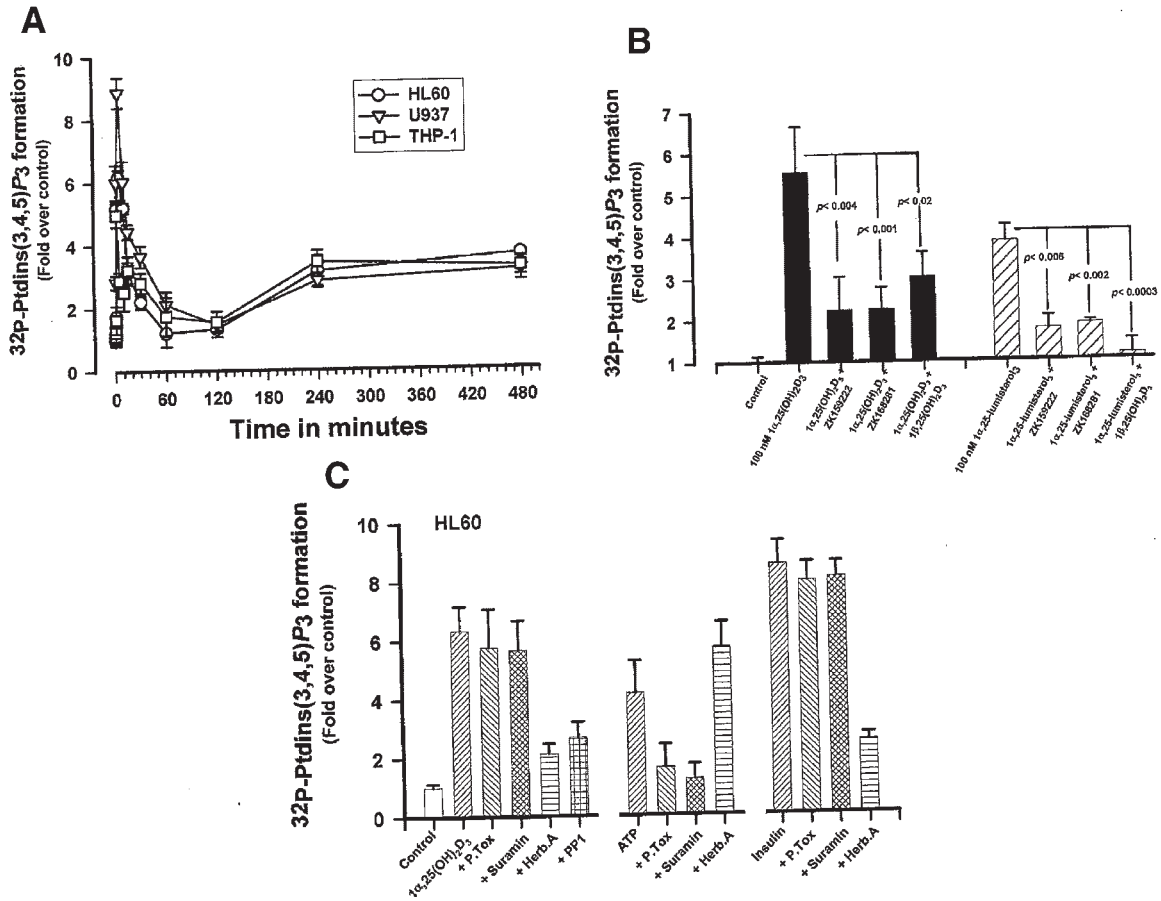

Fig. 1. 1α,25-Dihydroxyvitamin D_3 stimulates tyrosine kinase-activated, but not heterotrimeric G-protein-coupled, phosphatidylinositol 3-kinase isoforms in HL60, U937 and THP-1 myeloid leukaemic cells lines. **Panel A** shows the time course of the increase in PI3K activity in HL60 (○), U937 (▽) and THP-1 (□) cells after stimulation with 100 nM $1\alpha,25(OH)_2D_3$. **Panel B**: The $1\alpha,25(OH)_2D_3$- (100 nM, black column) or 1,25-dihydroxylumisterol$_3$- (100 nM, hatched column) stimulated increase in phosphatidylinositol 3-kinase activity in serum-starved HL60 cells was inhibited by the genomic antagonists ZK159222 (500 nM) or ZK168281 (500 nM) and the non-genomic antagonist $1\beta,25(OH)_2D_3$ (500 nM). Cells were treated with the antagonist for 30 min prior to agonist stimulation. Cells were then stimulated with the agonist under test for a further 15 min. **Panel C** shows the effects of the general tyrosine kinase inhibitor herbimycin A (50 μM), the Src family tyrosine kinase inhibitor PP1 (25 μM) or the heterotrimeric G-proteins inhibitors pertussis toxin (2 $\mu g\,ml^{-1}$) or suramin (100 μM) on $1\alpha,25(OH)_2D_3$-, insulin- and ATP-stimulated increase in phosphatidylinositol 3-kinase activity in serum-starved HL60 myeloid leukaemic cells. The cells were treated with the inhibitors for 1 h prior to stimulation. Cells were then stimulated with the agonist under test for a further 15 min. In each set of experiments the cells were labelled with ^{32}P and phosphoinositides were extracted, separated and quantitated as described in the Experimental Procedures Section. The data are presented as the mean ± s.e.m. of the fold increase in ^{32}P-PtdIns(3,4,5)P_3 production over control and were obtained in three experiments each performed in duplicate.

IA PI3K isoforms and/or PI3Kγ in myeloid leukaemic cells. We compared the properties of the $1\alpha,25(OH)_2D_3$-stimulated activation of PI3K in myeloid leukaemic cells with agents that activate PI3K via the tyrosine kinase or the heterotrimeric G-protein routes. HL60 and other myeloid cells express insulin receptors [Lord et al., 1988], which are coupled to the PI3K/Akt signalling pathway [Liu et al., 1998]. In most cell types, insulin and IGF-1 activate multiple class IA PI3K isoforms (usually predominately PI3Kα with varying contributions PI3Kβ or PI3Kδ), in a tyrosine kinase dependent manner [Chaussade et al., 2007]. In contrast, the nucleotide ATP potentially activates the class IA PI3Kβ isoform and class IB PI3Kγ via heterotrimeric G-protein coupled P2Y purinoceptors [Cosemans et al., 2006]. To investigate which class of PI3K is activated by $1\alpha,25(OH)_2D_3$ and other activators of PI3K,

myeloid leukaemic cell lines were treated with the general tyrosine kinase inhibitor herbimycin A (50 μM), the Src family tyrosine kinase inhibitor PP1 (25 μM) or the heterotrimeric G-proteins inhibitors pertussis toxin (2 μg ml^{-1}) or suramin (100 μM) for 1 h prior to stimulation.

Figure 1C shows that exposure of ^{32}P-labelled HL60 cells to 100 nM 1α,25$(OH)_2D_3$ produced a sixfold increase in the level of PtdIns(3,4,5)P_3 within 10 min of challenge. This increase was blocked by both herbimycin A and PP1, but was not significantly affected by suramin or pertussis toxin. ATP (1 μM) stimulated a ~4-fold increase in PI3K activity in HL60 cells that was blocked by pre-treatment with pertussis toxin and suramin, but was unaffected by herbimycin A (Fig. 1C). The insulin (5 μg/ml)-stimulated increase in PI3K activity in HL60 cells was completely blocked by herbimycin A and was unaffected by inhibitors of heterotrimeric G-protein signalling (Fig. 1C). Similar results were obtained using U937 and THP-1 cells (data not shown).

The p85 regulatory subunit of PI3K interacts, via its SH2 domain, with phosphotyrosine motifs on activated receptor tyrosine kinases or other accessory proteins, such as IRS-1 [Songyang et al., 1993]. The cell permeant peptide *N*-acetyl-Asp-Tyr(2-malonyl)-Val-Pro-Met-leu-NH_2 (PI3K-SH2-OMT) mimics these tyrosine phosphorylated binding targets of p85 and prevents activation of PI3K by agonists whose receptors are either directly or indirectly linked to tyrosine kinases [Ye et al., 1995]. Figure 2A shows that PI3K-SH2-OMT (25 μM) blocked the early increase in PI3K activity in ^{32}P-labelled HL60, U937 and THP-1 cells stimulated by 100 nM 1α,25$(OH)_2D_3$. Similarly, the insulin-stimulated increases in PI3K activity in HL60, U937 and THP-1 cells were blocked by PI3K-SH2-OMT. However, PI3K-SH2-OMT failed to have any effect on ATP-stimulated PI3K activity in each of the myeloid leukaemic cell lines. As an alternate approach we used U937 cells (U937:Δp85-clone18 cells) which when treated with TPA/Zn^{2+}/IPTG over-express a truncated dominant negative p85 subunit (data not shown). In uninduced U937:Δp85-clone18 cells, 1α,25$(OH)_2D_3$ (100 nM), insulin (5 μg/ml) and ATP (1 μM) all produced a modest two- to fivefold increases in PI3K activity (Fig. 2B). When expression of the dominant negative p85 subunit (Δp85) was induced, the increases in PI3K activity induced by 1α,25$(OH)_2D_3$ and insulin were severely blunted, whilst the response to ATP was essentially unaffected (Fig. 2B).

The 1α,25$(OH)_2D_3$-stimulated increase in steroid sulphatase activity was blocked by >60% ($p < 0.05$) by treating each of the myeloid leukaemic cells with 25 μM PI3K-SH2-OMT (Fig. 2C). Similarly, Figure 2D shows that the 1α,25$(OH)_2D_3$-stimulated increase in steroid sulphatase activity was inhibited by >75% ($n = 16$, $P > 0.00001$) following induction of expression of the dominant negative p85 subunit. Therefore, blockade of the association of the p85 with its tyrosine phosphorylated target proteins is sufficient to significantly impair the ability of 1α,25$(OH)_2D_3$ to stimulate an increase in steroid sulphatase activity. These results are consistent with the involvement of one of the class IA PI3K isoforms.

To identify the p110 catalytic subunit recruited to p85α THP-1 cells were loaded with an antibody specific for either p110α, p110β or p110δ using the Chariot^{TM} protein transfection reagent. THP-1 cells were used in this experiment because the transfection efficiency was low (consistently below 40%) in HL60 or U937 and <10–20% of these cells survived the transfection process. Antibody loaded THP-1 cells were then labelled with ^{32}P and stimulated with 1α,25$(OH)_2D_3$. Figure 3A shows that in the sham-loaded cells 100 nM 1α,25$(OH)_2D_3$ increased PI3K activity by 4.1 ± 0.32-fold over basal (○, $n = 3$, $P = 0.002$). There was a great deal of variation in the degree of 1α,25$(OH)_2D_3$-stimulated increase in PI3K activity in p110α antibody loaded cells, ranging from 1.27 to 3.42 fold over basal (▽, mean $= 2.23 \pm 0.25$, $n = 7$, $P = 0.0002$). The variability of the response is related to the efficiency of transfection because if the fold increase in 1α,25$(OH)_2D_3$-stimulated PI3K activity is plotted against the efficiency of transfection a clear inverse correlation emerges ($r^2 = 0.88$, Fig. 3B). In p110β antibody-loaded cell samples only a modest decrease in 1α,25$(OH)_2D_3$-stimulated PI3K activity (□, 3.27 ± 0.12-fold, $n = 4$, $P = 0.055$, Fig. 3B) was seen, even though transfection efficiency was greater than 60% in each sample. The 1α,25$(OH)_2D_3$-stimulated PI3K activity was unaltered in p110δ antibody loaded cells (◇, 3.78 ± 0.18-fold, $n = 4$, $P = 0.72$, Fig. 3A). These data show that 1α,25$(OH)_2D_3$-mediated activation of PI3Kα generates the bulk of the

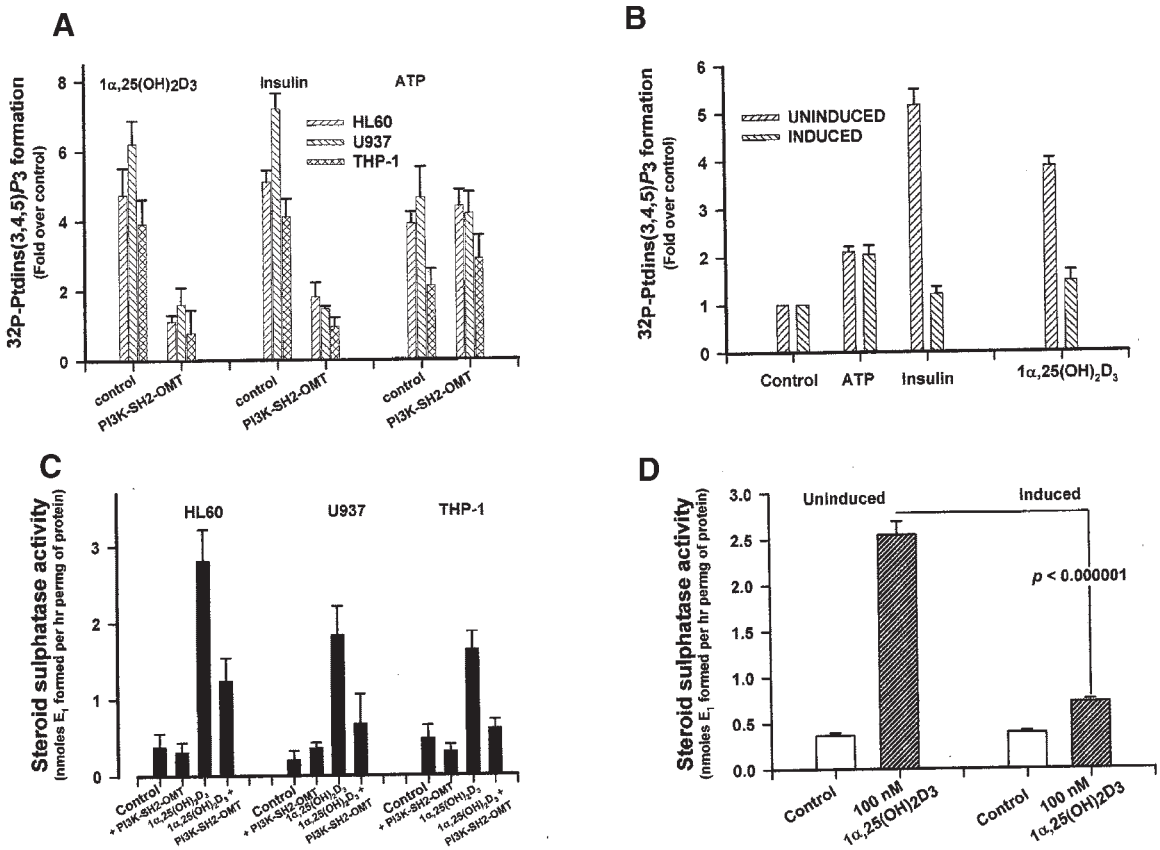

Fig. 2. 1α,25-Dihydroxyvitamin D_3 preferentially activates a tyrosine-kinase activated phosphatidylinositol 3-kinase in myeloid leukaemic cells. **Panel A** shows that the cell permeable peptide *N*-acetyl-Asp-Tyr(2-malonyl)-Val-Pro-Met-leu-NH_2 (PI3K-SH2-OMT) blocks $1\alpha,25(OH)_2D_3$—and insulin—but not ATP-stimulated PI3K activity in HL60 cells. HL60 cells were incubated with 25 μM PI3K-SH2-OMT for 60 min prior to stimulation with 100 nM $1\alpha,25(OH)_2D_3$, 5 μg/ml insulin or 1 μM ATP for 15 min. **Panel B** shows that $1\alpha,25(OH)_2D_3$ and insulin but not ATP failed to stimulate PI3K activity in induced U937:Δp85-clone 18 cells. When 20 mM IPTG, 1 nM PMA and 100 μM $ZnCl_2$ for 16 h U937:Δp85-clone 18 cells over-express a truncated dominant-negative p85 subunit (Δp85). Inducing agents were removed just before cells were stimulated with 100 nM $1\alpha,25(OH)_2D_3$, 5 μg/ml insulin or 1 μM ATP for 15 min. For the experiments shown in panels A and B the cells were labelled with ^{32}P and phosphoinositides were extracted, separated and quantitated as described in the Experimental Procedures Section. In each experiment the data are presented as the mean ± s.e.m. of the fold increase in ^{32}P-PtdIns(3,4,5)P_3 production over control and was obtained in four experiments performed in triplicate. **Panel C** shows that the cell permeable peptide *N*-acetyl-Asp-Tyr(2-malonyl)-Val-Pro-Met-leu-NH_2 (PI3K-SH2-OMT) blocks $1\alpha,25(OH)_2D_3$—and insulin—but not ATP-stimulated increase in steroid sulphatase activity in HL60 cells, U937 and THP-1 myeloid leukaemic cells. Cells were incubated with 25 μM PI3K-SH2-OMT for 60 min prior to stimulation with 100 nM $1\alpha,25(OH)_2D_3$, 5 μg/ml insulin or 1 μM ATP for 72 h. **Panel D** shows that failed to stimulate an increase in steroid sulphatase activity in Δp85 induced U937:Δp85-clone 18 cells. The expression of Δp85 was induced in U937:Δp85-clone 18 cells as described in panel A. Aliquots of induced and uninduced U937:Δp85-clone 18 cells were stimulated with 100 nM $1\alpha,25(OH)_2D_3$ for 72 min. Panels C and D show the steroid sulphatase activity recovered from the microsomal fraction and was measured as described in the Experimental Procedures Section. Results are shown as the mean ± s.e.m. steroid sulphatase activity expressed as the nmoles of E_1 formed per hr per mg of protein. The data shown in panel C was obtained in three experiments performed in triplicate. The data shown in panel D was obtained in 16 experiments performed in triplicate.

early rise in Ptds(3,4,5)P_3 levels in THP-1 cells. A minor contribution is made by PI3Kβ and there is none at all from PI3Kδ.

The role of PI3Kα in $1\alpha,25(OH)_2D_3$-stimulated increase in PI3K activity was confirmed in a THP-1 cell line (THP-1:HR-p110α3) in which expression of the p110α catalytic subunit of PI3K was silenced by RNAi. Figure 3C shows that an increase in PI3K activity was induced in THP-1:HR-mock cell line by $1\alpha,25(OH)_2D_3$, insulin and ATP. In the silenced THP-1:HR-p110α3 cell line only ATP produced the expected increase in PI3K activity and both the $1\alpha,25(OH)_2D_3$- and insulin-stimulated increases in PI3K activity were reduced by >70%.

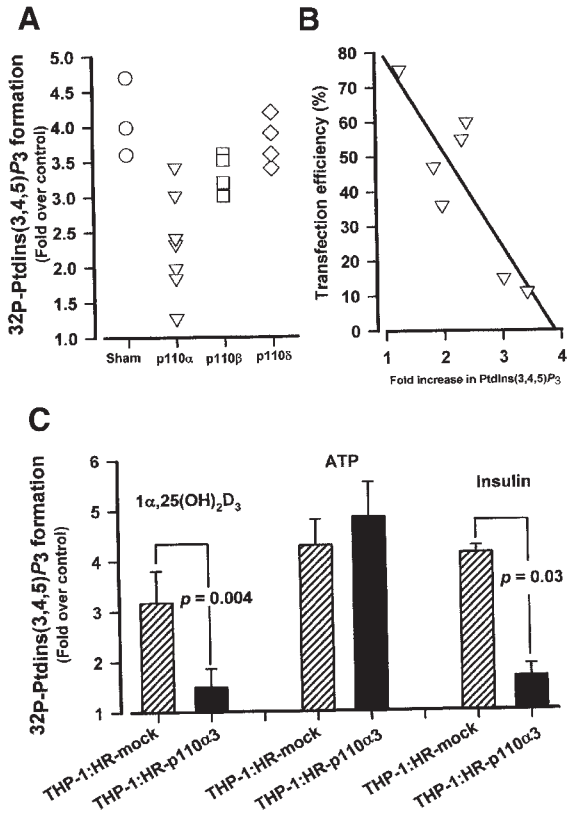

Fig. 3. 1α,25-dihydroxy vitamin D_3 preferentially activates PI3Kα in myeloid leukaemic cells. **Panel A**: THP-1 cells (5×10^5) were loaded with p110α (▽), p110β (□) and p110δ (◇) antibodies (5 μg) and β-galactosidase (1 μg) using the Chariot™ protein delivery reagent as described in the Experimental Procedures Section. The antibody loaded cells were labelled with ^{32}P, stimulated with 100 nM 1α,25$(OH)_2D_3$ for 15 min. Phosphoinositides were extracted, separated and quantitated as described in the Experimental Procedures Section. The data are presented as the mean fold increase in ^{32}P-PtdIns(3,4,5)P_3 production over control, each experiment was performed in duplicate. **Panel B** shows a plot of the mean fold increase in ^{32}P-PtdIns(3,4,5)P_3 production over control produced by stimulating p110α antibody loaded-THP-1 cells stimulated with 100 nM 1α,25$(OH)_2D_3$ versus the efficiency antibody loading. Transfection efficiency was assessed by estimating the number of cells expressing β-galactosidase activity and was measured essentially as described in the manufacturers recommendations. **Panel C** shows the mean fold increase in ^{32}P-PtdIns(3,4,5)P_3 production over control produced by stimulating THP-1:HR-p110α3 cells (black columns) in which expression of the p110α catalytic subunit of PI3K was silenced by RNAi [Lee et al., 2004] or THP-1:HR-mock cells (hatched columns) with either 100 nM 1α,25$(OH)_2D_3$, ATP (1 μM) or insulin (5 μg/ml). Cells were then stimulated with the agonist under test for 15 min. The cells were labelled with ^{32}P and phosphoinositides were extracted, separated and quantitated as described in the Experimental Procedures Section. The data are presented as the mean ± s.e.m. of the fold increase in ^{32}P-PtdIns(3,4,5)P_3 production over control and was obtained in four experiments performed in duplicate.

1α,25-Dihydroxyvitamin D_3-Stimulated PI3Kα Increases Phosphorylation of AKT in HL60 Cells

Figure 4A shows that the ratio of activated Akt phosphorylated on (serine-473) to total cellular following stimulation of HL60 cells with 100 nM 1α,25$(OH)_2D_3$. Akt activity was increased following stimulation of HL60 cells with 1α,25$(OH)_2D_3$, becoming significant within 15 min of the addition of the secosteroid and persisted for at least 96 h after stimulation. Zhang et al. [2006] have recently shown that 1α,25$(OH)_2D_3$ causes a similar persistent activation of Akt in HL60 cells. 1α,25$(OH)_2D_3$-stimulated increased in AKT phosphorylation (measured 60 min after stimulation) was dose responsive with an IC_{50} of ~0.9 nM and was blocked by the non-specific PI3K inhibitor LY294002 (Fig. 4B). Furthermore, the 1α,25$(OH)_2D_3$-stimulated increase in Akt phosphorylation was also significantly reduced by the genomic antagonist ZK159222 ($IC_{50} = 130 \pm 34$ nM) and the non-genomic antagonist 1β,25$(OH)_2D_3$ ($IC_{50} \sim 900$ nM, Fig. 4C). Figure 4D shows that 1α,25$(OH)_2D_3$ and insulin stimulated an increase in Akt phosphorylation (measured 60 min after stimulation) in the THP-1:HR-mock cell line but not in the silenced THP-1:HR-p110α3 cell line.

PI3Kα Is Involved in the 1α,25-Dihydroxyvitamin D_3-Stimulated Increase in Steroid Sulphatase Activity in HL60, U937 and THP-1 Myeloid Leukaemic Cells Lines

To investigate whether an increase in PI3K activity plays a role in the 1α,25$(OH)_2D_3$-stimulated increase in steroid sulphatase activity in myeloid cells HL60 cells were treated with small molecule inhibitors of PI3K, some of which show limited selectivity between PI3K isoforms, before 1α,25$(OH)_2D_3$-stimulation. Figure 5A shows that both wortmannin and LY294002 produced a dose responsive inhibition of 1α,25$(OH)_2D_3$-stimulated steroid sulphatase activity. These data reveal that PI3K is playing a role in the 1α,25$(OH)_2D_3$-stimulated in steroid sulphatase activity in myeloid leukaemic cells. However, neither wortmannin nor LY294002 are selective for individual PI3K isoforms and these experiments do not identify the particular class of PI3K that is involved in mediating this effect. Compounds that selectively inhibit individual p110 isoforms are becoming available [Hawkins

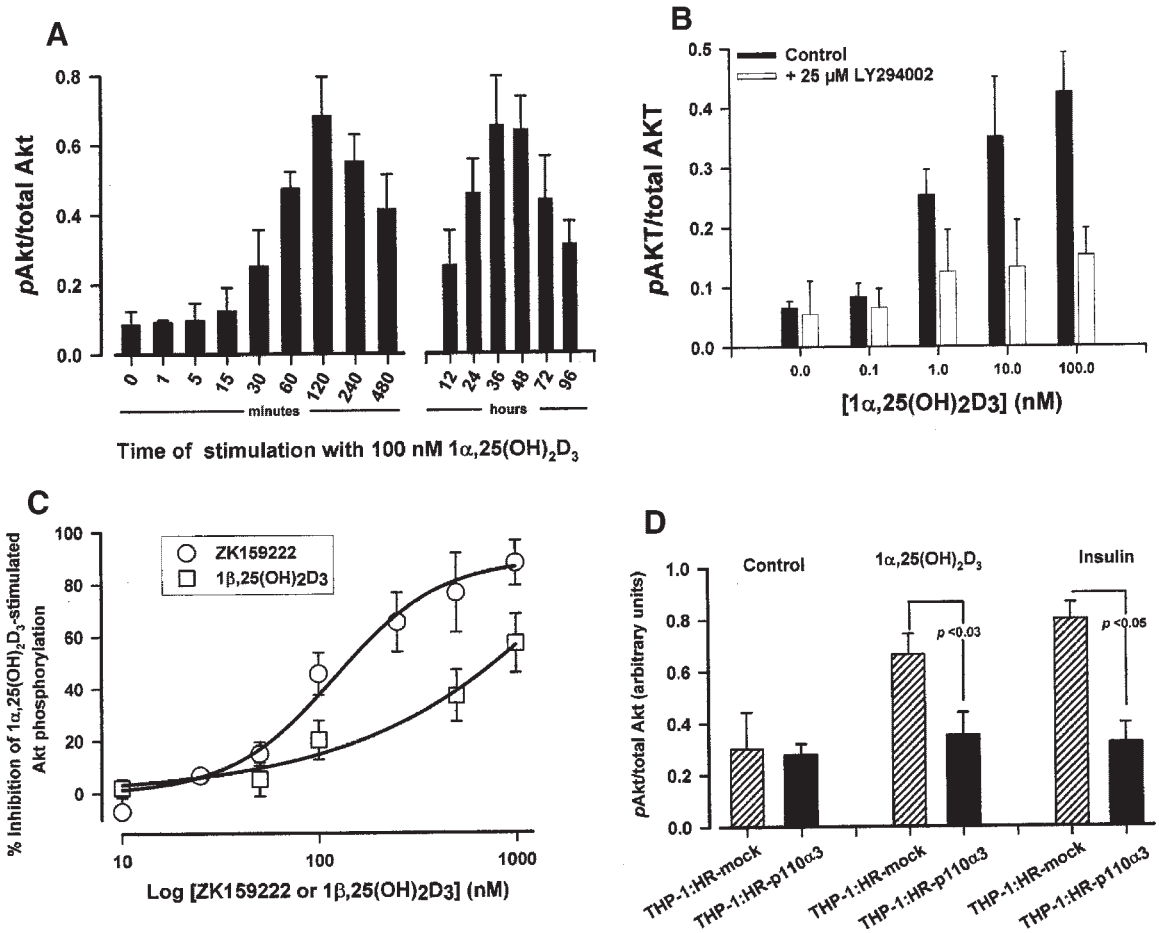

Fig. 4. 1α,25-Dihydroxyvitamin D_3 stimulated PI3Kα increases phosphorylation of AKT in HL60 cells. **Panel A** shows the time course of activation of Akt in HL60 cells stimulated with 100 nM $1\alpha,25(OH)_2D_3$. Data are shown as the ratio of activated (serine-473 phosphorylated) Akt (*p*Akt) to total cellular Akt. The normalised levels of pAkt and Akt were measured using the FACE™ AKT chemiluminescent Elisa assay kit (Actif Motif Europe, Rixensart, Belgium) essentially as described in the Experimental Procedures Section. The data are presented as the mean ± s.e.m. of the ration of *p*Akt/total Akt and was obtained in three experiments performed in triplicate. **Panel B** shows that the $1\alpha,25(OH)_2D_3$-stimulated Akt phosphorylation in HL60 cells was blocked by with the non-selective PI3K inhibitor LY294002. Cells were pre-treated with 25 μM LY294002 for 30 min prior to 60 min stimulation with 100 nM $1\alpha,25(OH)_2D_3$. The normalised levels of pAkt and Akt were measured essentially as described above. The data are presented as the mean ± s.e.m. of the ratio of *p*Akt/total Akt and was obtained in two experiments performed in triplicate. **Panel C** shows that $1\alpha,25(OH)_2D_3$-stimulated Akt phosphorylation in HL60 cells was blocked by both the genomic antagonist ZK159222 (○) or the non-genomic antagonist $1\beta,25(OH)_2D_3$(□). HL60 cells were pre-treated with 10–1,000 nM ZK159222 or 10–1,000 nM $1\beta,25(OH)_2D_3$ for 30 min prior to 60 min stimulation with 100 nM $1\alpha,25(OH)_2D_3$. The normalised levels of *p*Akt and Akt were measured essentially as described above. The data are presented as the mean ± s.e.m. of the ratio of *p*Akt/total Akt and was obtained in three experiments performed in triplicate. **Panel D** shows the effect of $1\alpha,25(OH)_2D_3$ or insulin on the activation of Akt in THP-1:HR-p110α3 cells (black columns) or THP-1:HR-mock cells (hatched columns). Cells were stimulated with either 100 nM $1\alpha,25(OH)_2D_3$, or insulin (5 μg/ml) for 60 min. The normalised levels of pAkt and Akt were measured using the FACE™ AKT chemiluminescent Elisa assay kit (Actif Motif Europe, Rixensart, Belgium) essentially as described in the Experimental Procedures Section. The data are presented as the mean ± s.e.m. of the ratio of *p*Akt/total Akt and was obtained in four experiments performed in triplicate.

et al., 2006]. For example, PX-866 [Ihle et al., 2005] and 3-[4-(4-morpholinyl)thioeno[3,2-d]pyrimidin-2-yl]-phenol (compound 15e) [Hayakawa et al., 2006] have recently been shown to be relatively specific PI3Kα inhibitors [Hawkins et al., 2006]. Figure 5A also shows that the $1\alpha,25(OH)_2D_3$-stimulated increase in steroid sulphatase activity in the HL60 cell line was inhibited by >75% by 10 μM PX-866 or 1 μM 3-[4-(4-morpholinyl)thioeno[3,2-d]pyrimidin-2-yl]-phenol. Similarly, at concentrations below 1 μM TGX-221 has been used as specific

PI3Kβ inhibitor in several haematopoietic cell systems [Condliffe et al., 2005; Jackson et al., 2005]. Figure 5A shows that the 1α,25$(OH)_2D_3$-stimulated increase in steroid sulphatase activity in the HL60 cell line was inhibited by ∼15% by 500 nM TGX-221. There are no commercially available high potency PI3Kδ specific inhibitors but the methylxanthine caffeine has been shown to be relatively specific inhibitor of PI3Kδ [$IC_{50} \sim 500$ μM vs. PI3Kδ compared with 10 mM vs. PI3Kα or PI3Kβ, Foukas et al., 2002]. Figure 5A shows that 1 mM caffeine inhibited 1α,25$(OH)_2D_3$-stimulated steroid sulphatase activity in the HL60 cell line by ∼10%. AS-252424 (up to 1 μM) has been shown to be a specific inhibitor PI3Kγ activation in intact myeloid leukaemic cells [Pomel et al., 2006]. Figure 5A shows that the 1α,25$(OH)_2D_3$-stimulated increase in steroid sulphatase activity in the HL60 cell line was essentially unaffected by pre-treatment with 5 μM AS-252424.

To confirm that PI3Kα plays a role in the 1α,25$(OH)_2D_3$-stimulated increase in steroid sulphatase activity we used THP-1 cells loaded with specific p110 isoform specific blocking antibodies. In this experiments aliquots of cells were selected so >60–70% of the cells contained the blocking antibody under test prior to stimulation with 1α,25$(OH)_2D_3$. Figure 5B shows that loading THP-1 cells with a p110α antibody reduced the 1α,25$(OH)_2D_3$-stimulated increase in steroid sulphatase activity by ∼70% ($n = 3$, $P < 0.01$). The 1α,25$(OH)_2D_3$-stimulated increase in steroid sulphatase activity was only marginally reduced in p110β- (∼25% inhibition, $n = 3$, $P = 0.12$) and was essentially unaffected in p110δ-loaded cells (∼10% inhibition, $n = 3$, $P = 0.77$).

Figure 5C shows that 100 nM 1α,25$(OH)_2D_3$ produced an ∼5-fold increase in steroid sulphatase activity in the sham transfected THP-1:HR-mock cell line whilst in transfected THP-1:HR-p110α3 cells steroid sulphatase activity was stimulated ∼ by less than 60% by 100 nM 1α,25$(OH)_2D_3$ ($n = 40$, $P < 0.0000005$). Similarly, the increases in steroid sulphatase activity stimulated by insulin and IGF-1 or SCF were severely attenuated in the THP-1:HR-p110α3 cell line when compared to the mock transfected control cell line ($n = 12$, $P < 0.0003$ and <0.0000005 respectively, Fig. 5D).

Taken together these observations show that 1α,25$(OH)_2D_3$-mediated activation of PI3Kα, rather than PI3Kβ or PI3Kδ, is involved in the secosteroid-stimulated increase in steroid sulphatase activity in myeloid cells.

The Effect of AKT Inhibitors on the 1α,25-Dihydroxyvitamin D_3-Stimulated Increase in Steroid Sulphatase Activity in HL60, U937 and THP-1 Myeloid Leukaemic Cells Lines

Several non-phosphorylatable phosphatidylinositol ether lipid analogues (e.g. 1-L-6-hydroxymethyl-*chiro*-inositol-2I-2-*O*-methyl-3-*O*-octadecylcarbonate (HIMO, Akt inhibitor I), SH-5 (Akt inhibitor II)) have been developed that act as either competitive inhibitors of the PI3K by mimicking the substrate PtdIns(4,5)P_2, or as competitive Akt inhibitors that prevent the membrane association of Akt by binding to its PH domain [Hu et al., 2000; Kozikowski et al., 2003]. Inhibitory cell-permeant 15-mer peptide TAT-Akt-*in* (Akt inhibitor VII) has also been developed that binds directly to the Akt PH domain [Hiromura et al., 2004]. Figure 6A shows that the 1α,25$(OH)_2D_3$-stimulated increase in Akt phosphorylation (measured 2 h after stimulation) was inhibited by >85% by 10 μM HIMO, 10 μM SH5 and 30 μM TAT-Akt-*in*. Matkovic et al. [2006] have shown that the retinoid-stimulated increase in Akt phosphorylation is inhibited by similar concentrations of these Akt inhibitors. Prolonged incubation (>48 h) of myeloid leukaemic cell lines with the Akt inhibitors produced unacceptably high levels of cytotoxicity (data not shown). Therefore, to assess the effects of the Akt inhibitors on the 1α,25$(OH)_2D_3$-stimulated increase in steroid sulphatase activity, the myeloid cell lines were incubated with increasing concentrations of Akt inhibitor for 60 min prior to stimulation with 100 nM 1α,25$(OH)_2D_3$. Steroid sulphatase activity was measured 24 h later. Using this protocol, the cell preparations were still ∼90–95% viable at the time when steroid sulphatase activity was evaluated. In each of the myeloid leukaemic cell lines the Akt inhibitors blocked the 1α,25$(OH)_2D_3$-stimulated increase in steroid sulphatase activity in a dose responsive fashion (HIMO, $IC_{50} \sim 0.5–5$ μM (Fig. 6B), SH5, $IC_{50} \sim 2–5$ μM (Fig. 6C), TAT-Akt-*in*, $IC_{50} \sim 10–50$ μM, Fig. 6D). It must be noted that the inhibition of the 1α,25$(OH)_2D_3$-stimulated increase in steroid sulphatase activity achieved by each inhibitor was approximately 80% of the maximum stimulation.

These data show that the PI3Kα-activated Akt plays an important role in the

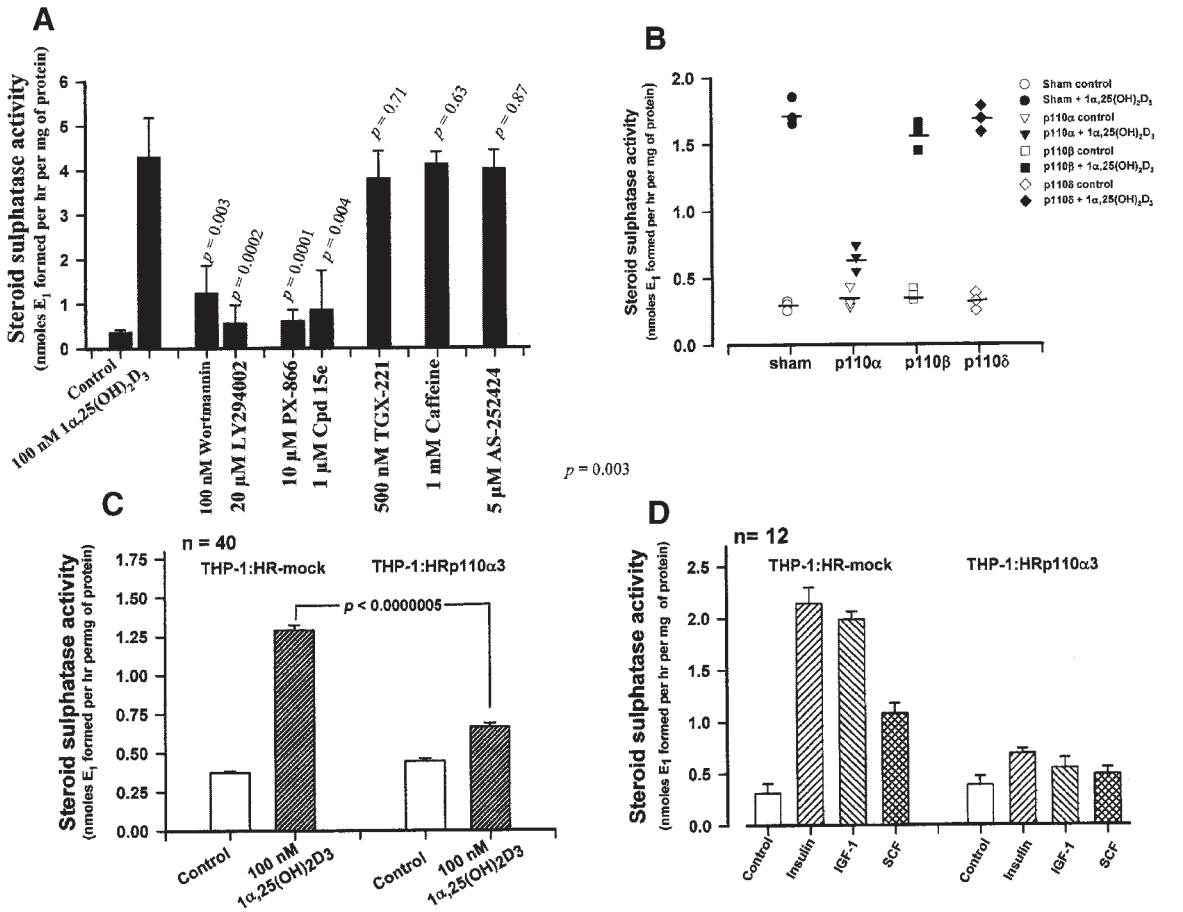

Fig. 5. PI3Kα mediates the 1α,25-dihydroxyvitamin D_3-stimulated increase in steroid sulphatase activity in myeloid leukaemic cells. **Panel A** the $1\alpha,25(OH)_2D_3$-stimulated increase in steroid sulphatase activity was abrogated in Hl60 cells by pharmacological inhibition of PI3K HL60 cells were incubated with the inhibitor under test for 60 min prior to stimulation with 100 nM $1\alpha,25(OH)_2D_3$. Microsomal steroid sulphatase activity was measured 72 h after stimulation as described in the Experimental Procedures Section. Results are presented as the mean ± s.e.m. steroid sulphatase activity expressed as the nmoles of E_1 formed per hr per mg of protein. The data were obtained in 3–9 experiments each performed in triplicate. **Panel B** shows that the $1\alpha,25(OH)_2D_3$-stimulated increase in steroid sulphatase activity was abrogated in THP-1 cells loaded with p110α antibody. THP-1 cells (5×10^5) were loaded 5 μg of either p110α (▽,▼), p110β (□,■) or p110δ (◇,◆) antibodies and 1 μg β-galactosidase using the Chariot™ protein delivery reagent as described in the Experimental Procedures Section. Three aliquots of cells were selected in which the transfection efficiency was greater 70%. These cell aliquots were stimulated with 100 nM $1\alpha,25(OH)_2D_3$ (filled symbols) or vehicle (open symbols) for 72 h. Microsomal steroid sulphatase activity was measured as described in the Experimental Procedures Section. Results are expressed as the mean steroid sulphatase activity expressed as the nmoles of E_1 formed per hr per mg of protein. The data were obtained in three cell aliquots and each determination was performed in triplicate. **Panel C** shows the increase in steroid sulphatase activity in THP-1:HR-p110α3 cells or THP-1:HR-mock cells (hatched columns) stimulated with 100 nM $1\alpha,25(OH)_2D_3$ (hatched columns) for 72 h. Microsomal steroid sulphatase activity was measured as described in the Experimental Procedures Section. Results are presented as the mean ± s.e.m. steroid sulphatase activity expressed as the nmoles of E_1 formed per hr per mg of protein. The data were obtained in 40 experiments each performed in triplicate. **Panel D** shows the increase in steroid sulphatase activity in THP-1:HR-p110α3 cells or THP-1:HR-mock cells (hatched columns) stimulated with insulin (5 μg/ml), IGF-1 (10 μg/ml) and rh-SCF (100 ng/ml) for 72 h. Microsomal steroid sulphatase activity was measured as described in the Experimental Procedures Section. The data are shown as the mean ± s.e.m. steroid sulphatase activity expressed as the nmoles of E_1 formed per hr per mg of protein. The data were obtained in 12 experiments each performed in triplicate.

$1\alpha,25(OH)_2D_3$-mediated stimulation of steroid sulphatase activity in myeloid leukaemic cell lines. A small fraction of the total $1\alpha,25(OH)_2D_3$-stimulated increase in steroid sulphatase activity was insensitive to both PI3K and Akt inhibitors, even at concentrations that completely blocked PI3K and Akt activation. Thus, other PI3K/Akt independent factors (e.g. the MAP kinase cascade [Hughes and Brown, 2006]) are also involved in the $1\alpha,25(OH)_2D_3$-mediated augmentation of steroid sulphatase activity.

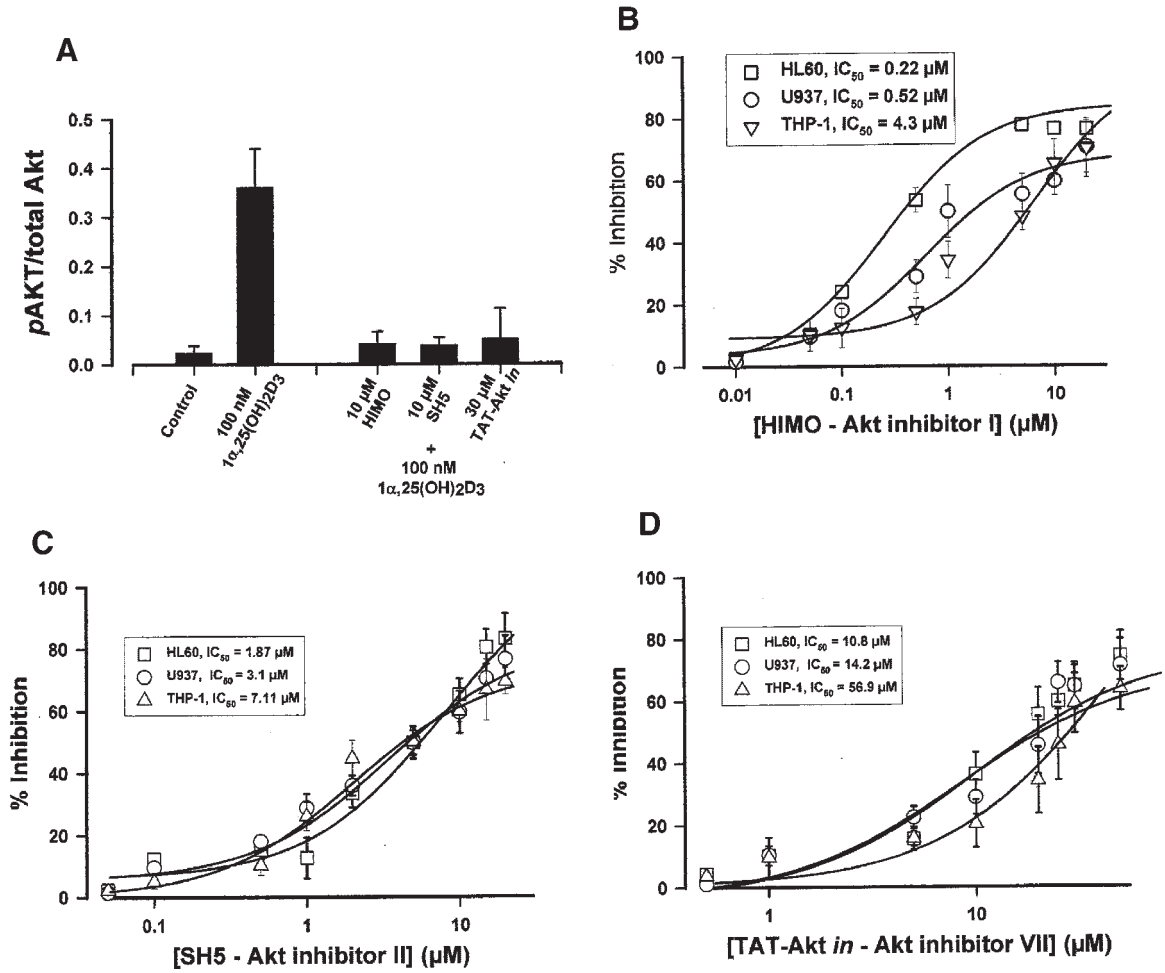

Fig. 6. Akt inhibitors abrogate the 1α,25-dihydroxyvitamin D_3-stimulated increase in steroid sulphatase activity in myeloid leukaemic cells. **Panel A** shows that the 1α,25$(OH)_2D_3$-stimulated increase in Akt phosphorylation in HL60 cells was blocked by HIMO (10 μM), SH5 (10 μM) and TAT-Akt-in peptide (30 μM). HL60 cells were pre-treated with the Akt inhibitors for 30 min prior to 60 min stimulation with 100 nM 1α,25$(OH)_2D_3$. The normalised levels of *p*Akt and Akt were measured essentially as described in the Experimental Procedures Section. The data are presented as the mean ± s.e.m. of the ratio of *p*Akt/total Akt and was obtained in three experiments each performed in triplicate. HL60 cells (□), U937 cells (○) or THP-1 cells (△) were treated with HIMO (0.01–20 μM, **panel B**), SH5 (0.05–25 μM, **panel C**), or TAT-Akt-in peptide (0.5–30 μM, **panel D**) for 30 min prior to stimulation with 100 nM 1α,25$(OH)_2D_3$. After 72 h microsomal steroid sulphatase activity was measured as described in the Experimental Procedures Section. Results are expressed as the mean ± s.e.m. inhibition of the increase in steroid sulphatase activity stimulated by 100 nM 1α,25$(OH)_2D_3$ alone. The data were obtained in six experiments each performed in triplicate. Inhibitory dose response curves were fitted to a sigmoidal dose-response curve (unrestricted Hill slope mode) and the IC_{50}'s and respective errors were calculated using the 'pharmacology' module of the Sigmaplot™ (version 9.0) graphical software package.

A number of downstream targets of Akt have been identified and validated in myeloid leukaemic cell lines. In HL60 cells the phosphorylation of glycogen synthase kinase-3β (GSK3β) is stimulated by 1α,25$(OH)_2D_3$ in a PI3K/Akt-dependent manner [Zhang et al., 2006]. The kinase activity of GSK3β is inactivated by phosphorylation. However, inhibition of GSK3β with the monovalent cation lithium chloride (2 mM) or indirubin-3′-monoximine (1 μM) failed to have any effect on either basal or the 1α,25$(OH)_2D_3$-mediated stimulation of steroid sulphatase activity in either HL60, U937 and THP-1 cells (data not shown). The antimycobacterial activity of 1α,25$(OH)_2D_3$ in human monocytic cells involves the generation of reactive oxygen species (ROS) by the PI3Kα/Akt-dependent activation of phagocytic

NADPH-dependent oxidase [Sly et al., 2001]. Pre-treatment of cells with H_2O_2 did not have any effect on either basal or 1α,25$(OH)_2D_3$-stimulated steroid sulphatase activity in HL60, U937 or THP-1 cells (data not shown). Prevention of the generation of ROS by the specific NADPH-dependent oxidase inhibitor diphenyleneiodonium chloride (5 μM), the specific xanthine oxidase inhibitor allopurinol (5 μM) or rotenone (10 μM), an inhibitor of mitochondrial ROS production, did not have any effect on either basal or 1α,25$(OH)_2D_3$-stimulated steroid sulphatase activity in HL60, U937 or THP-1 cells (data not shown). Similarly, decreasing ROS accumulation with the antioxidant *N*-acetyl-L-cysteine (10 mM) had no effect on either basal or 1α,25$(OH)_2D_3$-stimulated steroid sulphatase activity in HL60, U937 or THP-1 cells (data not shown). The PI3K/Akt pathway has also been implicated in the induction and activation of nitric oxide synthases (NOS) in myeloid cells. Neither the nitric oxide donor nitroso-*N*-acetyl-L,D-penicillamine nor the NOS inhibitor N^G-nitro-L-arginine methyl ester had any effect on either basal or 1α,25$(OH)_2D_3$-stimulated steroid sulphatase activity in HL60, U937 or THP-1 cells (data not shown). In HL60 cells, p70S6K is phosphorylated in a PI3K/Akt-dependent fashion following 1α,25$(OH)_2D_3$ stimulation [Zhang et al., 2006]. 1α,25$(OH)_2D_3$-stimulated phosphorylation of p70S6K can be blocked by pretreating HL60 cells with the macrolide immunosuppressant rapamycin [Zhang et al., 2006]. Rapamycin (500–2,500 nM) had no effect on basal steroid sulphatase activity but did have a moderate inhibitory effect (<35%) on the 1α,25$(OH)_2D_3$-stimulated increase in steroid sulphatase in HL60 cells (data not shown). Therefore, an increase in translational efficiency appears to play a small role in the 1α,25$(OH)_2D_3$-stimulated increase in steroid sulphatase in myeloid cells.

Depending on the cell type investigated 1α,25$(OH)_2D_3$ is able to either stimulate or inhibit nuclear factor-κB (NF-κB) activity. Tse et al. [2007] have reported that 1α,25$(OH)_2D_3$ transiently inhibits NF-κB signalling in HL-60 cells; this is followed by a delayed prolonged period of activation. The late rise in NF-κB activity is paralleled by an increase in IκB kinase (IKK) activity, phosphorylation of the IκBα inhibitory subunit (i.e. leading to its inactivation following degradation in the proteosome), phosphorylation and nuclear translocation of the NF-κB p65(relA) subunit and p65 recruitment to the NF-κB/vitamin D responsive element promoters. NF-κB is a *bona-fide* PI3K/Akt target in HL60 cells since PI3K/Akt antagonists suppressed 1α,25$(OH)_2D_3$-stimulated IκBα phosphorylation as well as the expression of NF-κB-controlled genes. Figure 7 shows that pre-treatment of HL60 cells with the IKK inhibitors wedelolactone (20 μM) [Kobari et al., 2004; Tse et al., 2007], parthenolide (20 μM) [Hehner et al., 1999] and thalidomide (10 μM) [Keifer et al., 2001] blocked the 1α,25$(OH)_2D_3$-stimulated increase in steroid sulphatase in HL60, U937 and THP-1 cells by >90%. Similarly, Figure 7A also shows that preventing the proteosomal degradation of IκBα with the proteosome inhibitor MG-132 (10 μM) [Guzman et al., 2001] inhibited the 1α,25$(OH)_2D_3$-stimulated increase in steroid sulphatase activity in each myeloid leukaemic cell line by >75%. Finally, pre-treatment of the myeloid leukaemic cells with the cell permeable peptide SN50 (25 μM), which prevents translocation of the activated NF-κB complex into the nucleus [Lin et al., 1995], inhibited the 1α,25$(OH)_2D_3$-stimulated increase in steroid sulphatase activity in each myeloid leukaemic cell line by >80% (Fig. 7A).

High concentrations of dimethylsulphoxide (DMSO) induce granulocytic differentiation of HL60 cells [Chang et al., 2006]. At these 'differentiating' concentrations DMSO increase PI3K activity [Ai et al., 1995; Cataldi et al., 2006] and stimulate NF-κB-mediated transactivation in HL60 cells [Lee et al., 2005b]. In neuronal cells, SCF activates NF-κB by a PI3K/Akt-driven mechanism [Dhandapani et al., 2005]. The PI3Kα/Akt/NF-κB signalling cassette is stimulated by insulin and related compounds in a variety of haematopoietic cells [Jimenez Del Rio and Velez-Pardo, 2006]. Therefore, activation of the PI3K/Akt/NF-κB signalling cassette appears to be a common element in 1α,25$(OH)_2D_3$-, insulin-, stem cell factor- and DMSO-mediated signalling in HL60 cells. Figure 7B shows that steroid sulphatase activity was increased by two- to fivefold in HL60 cells following 72 h exposure to either insulin (5 μg/ml), recombinant human stem cell factor (100 ng/ml) or DMSO (1.5% (V/V)). The increase in steroid sulphatase activity produced by each agent was significantly reduced by pretreatment with the non-specific PI3K inhibitor

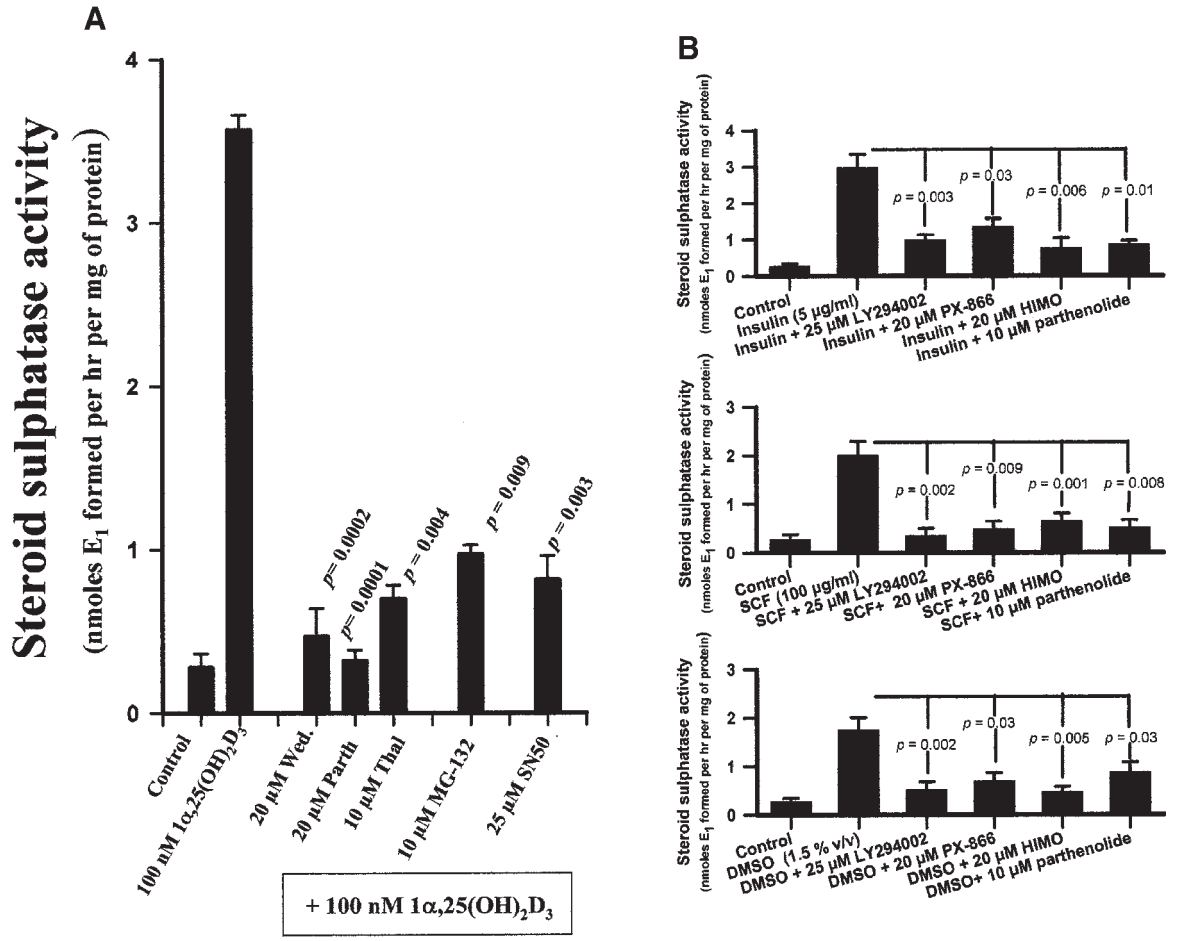

Fig. 7. NF-κB inhibitors abrogate the 1α,25-dihydroxyvitamin D_3-stimulated increase in steroid sulphatase activity in myeloid leukaemic cells. **A** shows that inhibitors of NF-κB signalling block the $1\alpha,25(OH)_2D_3$ stimulated increase in steroid sulphatase activity in HL60 cells. HL60 cells were treated with the IKK inhibitors (wedelolactone (20 μM), parthenolide (20 μM) and thalidomide (10 μM)), the proteosome inhibitor MG-132 (10 μM) of the cell permeable peptide SN50 (25 μM) for 60 min prior to stimulation with 100 nM $1\alpha,25(OH)_2D_3$. After 72 h microsomal steroid sulphatase activity was measured as described in the Experimental Procedures Section. Results are shown as the mean ± s.e.m. steroid sulphatase activity expressed as the nmoles of E_1 formed per hr per mg of protein. The data were obtained in 3–9 experiments each performed in triplicate. **B** shows that inhibition of the PI3K/Akt/NF-κB signalling cassette blocks insulin, stem cell factor and DMSO stimulated increase in steroid sulphatase activity in HL60 cells. HL60 cells were stimulated with insulin (5 μg/ml, **top panel**), recombinant human stem cell factor (100 ng/ml; **middle panel**) or DMSO (1.5% (V/V), **bottom panel**) for 72 h. Cells were incubated with either 25 μM LY294002, 20 μM PX-866, 20 μM HIMO or 10 μM parthenolide for 60 min prior to stimulation. Microsomal steroid sulphatase activity was measured as described in the Experimental Procedures Section. Results are shown as the mean ± s.e.m. steroid sulphatase activity expressed as the nmoles of E_1 formed per hr per mg of protein. The data were obtained in four experiments each performed in triplicate.

LY294002 (25 μM), the Akt inhibitor HIMO (20 μM) or the NF-κB inhibitor parthenolide (10 μM).

DISCUSSION

There is increasing evidence that the biological effects of nuclear hormones are mediated by a complex mix of directly controlled gene expression (genomic effects) and the parallel activation of cell signalling/phosphorylation cascades (i.e. non-genomic or extra-nuclear actions). The nature of the hormone receptors that mediate these direct transcription-independent membrane-initiated-signalling effects is currently the subject of much debate. It has been widely reported that the 'non-genomic' PI3K/Akt signalling cascade is activated by hormones, such as 17β-estradiol, thyroid hormone, all-*trans*-retinoic acid and testosterone, in a variety of cell types [Simoncini et al., 2000; Sun et al., 2003; Baron et al., 2004; Cao et al.,

2005; Furuya et al., 2006, 2007; Greger et al., 2007; Kim et al., 2007; Masiá et al., 2007]. Consequently, a mechanistic consensus is starting to emerge whereby a small pool of the nuclear receptor is either (1) permanently inserted into a cholesterol rich micro-domain (caveolae) in the plasma membrane, or (2) rapidly translocates to the plasma membrane upon binding its cognate ligand. At the membrane, the liganded nuclear receptors can associate with the p85 regulatory and p110 catalytic subunits of a member of the class IA PI3Ks family, effector molecules (such as the tyrosine kinase c-SRC) and scaffolding/adaptor proteins (e.g. MNAR, Grb2) to form a multimeric membrane-associated 'signalosome'.

In this article we shown that in HL60 cells the VDR_{nuc} plays a central role in the $1\alpha,25(OH)_2D_3$-mediated increase in PI3K activity. In HL60, and most other cell types, the bulk of the unliganded VDR_{nuc} is found in the cytosol. Following cell stimulation with $1\alpha,25(OH)_2D_3$ the VDR_{nuc} slowly translocates to the nucleus [Barsony and Prufer, 2002; Gocek et al., 2007; Klopot et al., 2006; Wu-Wong et al., 2006], where it is found in either discrete foci in the nuclear matrix or in distinct clusters in the nuclear membrane [Kivineva et al., 1998]. However, it has been estimated that 1–3% of cellular VDR_{nuc} is located in or around cholesterol-rich micro-domains of the plasma membrane in several cell types [Kim et al., 1996; Capiati et al., 2002; Huhtakangas et al., 2004]. In NB4 cells a small fraction of the total cellular VDR_{nuc} can be recovered from caveolae-rich membrane fraction [Huhtakangas et al., 2004], whilst in HL60 and other primary myeloid leukaemic cells a small pool of liganded VDR_{nuc}, associates with the F-actin cytoskeleton in close proximity to the plasma membrane [Gocek et al., 2007]. In myeloid cells components of the PI3K/Akt signalling cascade are found in the cytoplasm, cholesterol-rich microdomains in the plasma membrane [Seveau et al., 2001; Gomez-Mouton et al., 2004; Van Keymeulen et al., 2006], nuclear membrane and in nuclear speckles [Bertagnolo et al., 2004; Martelli et al., 2006; Matkovic et al., 2006]. Therefore, the classical VDR_{nuc} is found in the same cellular compartments as components of the PI3K/Akt signalling pathway in myeloid cells.

Experiments showing that the 'genomic' agonist ZK159222 blocks the $1\alpha,25(OH)_2D_3$-stimulated increase in PI3K activity suggest the involvement of VDR_{nuc}. There is some direct evidence that the VDR_{nuc} is involved in the activation of PI3K. Vertino et al. [2005] have used a variety of VDR_{nuc} truncation mutant proteins to show that an intact central ligand binding domain is necessary and sufficient for $1\alpha,25(OH)_2D_3$-mediated activation of PI3K in osteocytes. Preliminary experiments in HL60 cells have shown that following $1\alpha,25(OH)_2D_3$-stimulation PI3K activity can be recovered in VDR_{nuc}-immunoprecipitates (data not shown). The VDR_{nuc} and $1\alpha,25(OH)_2D_3$ are both conformationally flexible molecules. This conformational flexibility of both ligand and its receptor may explain why the liganded VDR_{nuc} can activate both genomic and non-genomic signalling pathways. A model developed in the Norman lab (Conformation ensemble model) suggests that the liganded-VDR_{nuc} can adopt separate conformations which can 'engage productively' with components of either the genomic or the non-genomic signalling pathways, [Norman et al., 2004; Norman, 2006]. To stimulate non-genomic signalling pathways, $1\alpha,25(OH)_2D_3$ must bind to the VDR_{nuc} in a 'basket-like' 6-*s-cis* configuration [Norman et al., 2004]. We show that both $1\alpha,25(OH)_2D_3$ and its '*6-s-cis* locked' analogue 1α,25-dihydroxylumisterol$_3$ can activate the PI3K signalling pathway in HL60 cells. The $1\alpha,25(OH)_2D_3$- and 1α,25-dihydroxylumisterol$_3$-stimulated increases in PI3K activity were blocked by the 'genomic antagonist' ZK159222 and the 'non-genomic antagonist' $1\beta,25(OH)_2D_3$. Trypsin digestion experiments have shown that the ZK159222 bound-VDR_{nuc} adopts a unique conformation [Castillo et al., 2006] and appears that this 'antagonistic' configuration of the receptor is unable to couple with essential components of both genomic and non-genomic signalling pathways. The non-genomic antagonist $1\beta,25(OH)_2D_3$ competes with $1\alpha,25(OH)_2D_3$ (in their *6-s-cis* conformations) and 1α,25-dihydroxylumisterol$_3$ for access to the non-genomic signalling pocket.

Which PI3K isoform(s) is/are stimulated by $1\alpha,25(OH)_2D_3$ in myeloid cells? In other cell types $1\alpha,25(OH)_2D_3$ signals via tyrosine kinase- [Boland et al., 2005] or heterotrimeric G-protein activated-signalling pathways [Le Mellay et al., 1999]. The heterotrimeric G-protein-activated class IB PI3Kγ is the most abundant PI3K isoform found in myeloid cells. However, both pertussis toxin treatment or transfection with a

p110γ blocking antibody failed to have any effect of the 1α,25$(OH)_2D_3$-stimulated increase in PI3K activity in myeloid cells. Myeloid cells also express all three tyrosine kinase activated class IA PI3Ks. The recruitment of the tyrosine kinase c-Src to the nascent signalosome appears to be a common feature in the activation of PI3K by nuclear hormone receptors. Activated c-Src then phosphorylates either the steroid hormone receptor itself or one of the other signalosome components (e.g. MNAR). This allows recruitment of the p85 regulatory subunit of PI3K which binds via an SH2 domain in its C-terminus, and activation of the p110 catalytic subunit. The VDR_{nuc} is found in a complex with c-Src in several 1α,25$(OH)_2D_3$-stimulated cell types [Buitrago et al., 2000]. In osteocytes small molecule inhibitors of c-Src block the 1α,25$(OH)_2D_3$-stimulated VDR_{nuc}-mediated increase in PI3K activity [Vertino et al., 2005]. We have shown that: (i) inhibition of Src family tyrosine kinases blocked 1α,25$(OH)_2D_3$-mediated activation of PI3K and Akt in myeloid cells (ii) treatment of cells with a cell permeant peptide N-PI3K-SH2-OMT, which competes with the SH2 domain of p85, blocked 1α,25$(OH)_2D_3$-mediated activation of PI3K and Akt in myeloid cells and, (iii) no increase in PI3K or Akt activity was observed in 1α,25$(OH)_2D_3$-stimulated U937:Δp85-clone18 cells once the expression of a truncated p85 subunit that competitively binds to phosphotyrosine target proteins and prevents activation of the catalytic subunit of PI3K was induced. These observations are consistent with the notion that one or more components of the VDR_{nuc} containing signalosome needs to contain a motif that can be phosphorylated by the Src tyrosine kinase thereby creating a domain that can be recognised by the SH2 domain of p85. Furthermore in THP-1 cells, the 1α,25$(OH)_2D_3$-stimulated increase in PI3K activity was inhibited in cells in which expression of the p110α catalytic subunit of PI3K was silenced by RNAi or by transfecting cells with blocking antibodies directed against PI3Kα but not PI3Kβ or δ.

Activation of PI3Kα and Akt by 1α,25$(OH)_2D_3$ has important functions in myeloid cells. Pharmacological inhibition of 1α,25$(OH)_2D_3$-stimulated PI3Kα activity block phagosome maturation and NADPH-dependent anti-mycobacterial activity in maturing myeloid leukaemic cell lines [Sly et al., 2001; Hmama et al., 2004]. Furthermore, activation of PI3K and Akt are essential to 1α,25$(OH)_2D_3$-driven myeloid differentiation and prevention of apoptosis. Here the use of pharmacological, biochemical and immunological tools have shown that an increase in PI3Kα and in Akt activities are required for the 1α,25$(OH)_2D_3$-stimulated increase in steroid sulphatase activity in myeloid cells. Of the downstream Akt targets examined, only the NF-κB signalling pathway played a significant role in the 1α,25$(OH)_2D_3$-stimulated increase in steroid sulphatase activity. Akt increases the nuclear accumulation of NF-κB and stimulates its transcriptional activity in a variety of cell types [Manning and Cantley, 2007]. It has been suggested that that the mechanism of activation involves direct phosphorylation of the amino acid residue T23 on IκB kinase α (IKKα) [Ozes et al., 1999]. Several lines of evidence suggest that PI3K/Akt cascade stimulates NF-κB in HL60 cells. An HL60 cell subline that is resistant to pro-apoptotic stimuli has constitutive expression of activated Akt1, NF-κB is persistently translocated to the nucleus, and expression of several NF-κB-controlled pro-survival genes are up-regulated [Bortul et al., 2003]. In the related NB4 cell line, 1α,25$(OH)_2D_3$-stimulates the phosphorylation of the IκBα regulatory subunit and translocation of the active NF-κB transcription factor complex to the nucleus [Berry et al., 2002]. This process is necessary for monocytic differentiation of these cells.

In HL60 cells, the effect of 1α,25$(OH)_2D_3$ on NF-κB activity is more complicated. During the first 4–8 h after 1α,25$(OH)_2D_3$ stimulation NF-κB activity is transiently inhibited, due to increased expression of the regulatory IκBα subunit. Around 12 h after addition of 1α,25$(OH)_2D_3$ NF-κB activity begins to increase. This is dependent on Akt-dependent IKK-mediated phosphorylation and degradation of the regulatory IκB [Tse et al., 2007]. This study, and others, show that in 1α,25$(OH)_2D_3$-stimulated HL60 cells Akt activation is slow to start but lasts for many h. This is consistent with the time course of NF-κB activation in HL60 cells [This paper, Zhang et al., 2006; Tse et al., 2007]. Furthermore, we have previously shown in HL60 cells that there is a 12–16 h delay after 1α,25$(OH)_2D_3$ stimulation before any increase in steroid sulphatase activity is observed [Hughes et al., 2001]. Again, the time course of the increase in steroid sulphatase

activity is consistent with the kinetics of $1\alpha,25(OH)_2D_3$-stimulated Akt-mediated NF-κB activation in HL60 cells. Consistent with these observations, we have shown that inhibition of IKK activity, IκB degradation of p50/p65 nuclear translocation NF-κB in HL60 and other myeloid cell lines had an inhibitory effect on $1\alpha,25(OH)_2D_3$-stimulated steroid sulphatase activity.

We have previously shown that the retinoid-stimulated increase in steroid sulphatase activity/expression in HL60 cells is partially dependent on PI3K/Akt activation of the NF-κB [Hughes et al., 2005]. A role for NF-κB in the regulation of steroid sulphatase activity/expression can also inferred from the following observations. Steroid sulphatase activity/expression is higher in the malignant, highly metastatic breast cancer MDA-MB-231 cell line when compared with the less aggressive MCF-7 cell line [Hughes, unpublished work; Purohit and Reed, 1992; Selcer et al., 1997; Ng et al., 2000; Chong et al., 2006; Selcer et al., 2007]. Both constitutive and agonist stimulated PI3K/Akt and NF-κB pathways are constitutively activated in MDA-MB-231 cells relative to MCF-7 cells. [Monks and Pardee, 2006; Nakshatri et al., 1997; Sliva et al., 2002; Upadhyay et al., 2006]. Preliminary experiments have shown that pharmacological blockade of NF-κB signalling reduces steroid sulphatase activity/expression in MDA-MB-231 cells (Hughes and Brown, manuscript in preparation). Recently, Gareus et al. [2007] generated a mouse in which IKKα activity has been specifically knocked-out in keratinocytes. Basal steroid sulphatase activity and expression in the keratinocytes is much reduced in mice in keratinocytes from these mice [Gareus et al., 2007].

In conclusion, we shown that: (i) $1\alpha,25(OH)_2D_3$, acting via the VDR_{nuc}, stimulates PI3Kα and Akt activity in myeloid cells, and (ii) activation of the PI3Kα/Akt/NF-κB signalling pathway makes a significant contribution to the $1\alpha,25(OH)_2D_3$-stimulated increase in steroid sulphatase activity. The latter observation may be pertinent to the development of some hormone-dependent cancers. PI3Kα [Foukas et al., 2006; Perez-Tenorio et al., 2007] and NF-κB signalling [Zhou et al., 2005] pathways are often activated in many breast cancers. It is tempting to speculate that this may contribute to the elevated steroid sulphatase activity observed in many breast tumours. An elevated steroid sulphatase activity/expression has been observed in patients with endometriosis [Yanaihara et al., 2005], adenomyosis [Kitawaki, 2006], as well as ovarian and endometrial [Tanaka et al., 2003; Utsunomiya et al., 2004; Smuc et al., 2005] and breast [Suzuki et al., 2005; Chong et al., 2006; Sasano et al., 2006; Tsunoda et al., 2006] cancers. There is a strong correlation between steroid sulphatase activity/expression and the prognosis of post-menopausal women with breast cancer [Suzuki et al., 2005; Chong et al., 2006; Sasano et al., 2006; Tsunoda et al., 2006]. A recent phase I clinical trial has shown that bioactive estrogen production within breast tumours and stabilises disease progression in post-menopausal patients with estrogen-dependent tumours was reduced by pharmacological inhibition of steroid sulphatase activity [Stanway et al., 2006]. An increased understanding of how the PI3Kα/Akt/NF-κB signalling pathway controls the expression/activity of steroid sulphatase activity may provide novel approaches to the diagnosis and treatment of estrogen/androgen-dependent tumours of many tissues.

ACKNOWLEDGMENTS

This work was funded by a project grants awarded to PJH and GB by the Leukaemia Research Fund (grant no. LRF-0257) and to NER by the Canadian Institutes of Health Research (grant no. MOP-8633). Grateful thanks are extended to Dr. Lise Binderup (Leo Pharmaceutical Products, Ballerup, Denmark) for her gift of $1\alpha,25$-dihydroxyvitamin D_3, Dr. Andreas Steinmeyer (Schering-Plough AB, Berlin, Germany) for supplying ZK159222 and ZK168281 and Dr Roshantha Chandraratna (Vitae Pharmaceuticals, Irvine, CA) for the gift of VTP195393. Dr. Phillip Hawkins of the inositide signalling laboratory (The Babraham Institute, Cambridge, UK) is thanked for supplying the U937:Δp85-p18 cells.

REFERENCES

Ai Z, Misra S, Susa M, Variticovski L, Cohen C. 1995. Phosphatidylinositol 3-kinase activity in murine erythroleukemia cells during DMSO-induced differentiation. Exp Cell Res 219:454–460.

Baron S, Mamin M, Beaudoin C, Leotoing L, Communal L, Veyssiere G, Morel L. 2004. Androgen receptor mediates non-genomic activation of phosphatidylinositol 3-OH kinase in androgen-sensitive epithelial cells. J Biol Chem 279:14579–14586.

Barsony J, Prufer K. 2002. Vitamin D receptor and retinoid X receptor interactions in motion. Vitam Horm 65:345–376.

Berry D, Clark C, Meckling-Gill K. 2002. 1α,25-dihydoxyvitamin D_3 stimulates phosphorylation of IκBα and synergises with TPA to induce nuclear translocation of NF-κB during monocytic differentiation of NB4 leukaemia cells. Exp Cell Res 272:176–184.

Bertagnolo V, Brugnoli F, Marchiso M, Capitano S. 2004. Inositide-modifying enzymes: A cooperative role in regulating nuclear morphology during differentiation of myeloid cells. J Biol Regul Homeost Agents 18:381–386.

Boland R, Buitrago C, De Boland A. 2005. Modulation of tyrosine phosphorylation signalling pathways by 1α,25 $(OH)_2$-vitamin<D. Trends Endocrinol Metab 16:280–287.

Bortul R, Tazzari P, Cappellini A, Tabellini G, Billi A, Bareggi R, Manzoli L, Cocco L, Martelli A. 2003. Constitutively active Akt1 protects HL60 leukaemia cells from TRAIL-induced apoptosis through a mechanism involving NF-κB activation and cFLIP(L) up-regulation. Leukaemia 17:379–389.

Buitrago C, Vazquez G, De Boland A, Boland R. 2000. Activation of src kinase in skeletal muscle cells by 1,25$(OH)_2$-vitamin D_3 correlates with tyrosine phosphorylation of the vitamin D receptor (VDR) and VDR-Src interaction. J Cell Biochem 79:274–281.

Cao X, Kambe F, Moeller L, Refetoff S, Seo H. 2005. Thyroid hormone induced rapid activation of Akt/protein kinase B-mammalian target of rapamycin-$p70^{S6K}$ cascade through phosphatidylinositol 3-kinase in human blasts. Mol Endocrinol 19:102–112.

Capiati D, Benassati S, Boland R. 2002. 1α,25$(OH)_2$-vitamin D_3 induces translocation of the vitamin D receptor (VDR) to the plasma membrane in skeletal muscle cells. J Cell Biochem 86:128–135.

Castillo A, Sanchez-Martinez R, Jimenez-Lara A, Steinmeyer A, Zugel U, Aranda A. 2006. Characterization of vitamin D receptor ligands with cell-specific and dissociated activity. Mol Endocrinol 20:3093–3104.

Cataldi A, Di Pietri R, Centurione L, Grilli A, Miscia S. Phosphatidylinosotol 3-kinase activation and atypical protein kinase Cζ phosphorylation characterise the DMSO signalling in erythroleukaemia cells. Cell Signal 2000. 12:667–672.

Chang H, Oh P, Ingber D, Huang S. 2006. Multistable and multistep dynamics in neutrophil differentiation. BMC Cell Biol 7:11.

Chaussade C, Rewcastle G, Kendall J, Denny W, Cho K, Gronning L, Chong M, Anagnostou S, Jackson S, Daniele N, Sheperd P. 2007. Evidence for functional redundancy in class-IA PI3-kinase isoforms in insulin signalling. Biochem J 404:449–458.

Chong Y, Colston K, Jiang W, Sharma A, Mokbel K. 2006. The relationship between the insulin-like growth factor-1 system and the oestrogen metabolising enzymes in breast cancer tissue and its adjacent non-cancerous tissue. Breast Cancer Res Treat 99:275–288.

Condliffe A, Davidson K, Anderson K, Ellson C, Crabbe T, Okkenhaug K, Vanhaesebroeck B, Turner M, Webb L, Wymann M, Hirsch E, Ruckle T, Camps M, Rommel C, Jackson S, Chilvers E, Stephens L, Hawkins P. 2005. Sequential activation of class 1B and class 1A PI3K is important for the primed respiratory burst of human but not murine neutrophils. Blood 106:1432–1440.

Cosemans J, Munnix I, Wetzker R, Heller R, Jackson S, Heemskerk J. 2006. Continuous signalling via PI3K isoforms β and γ is required for platelet ADP receptor function in dynamic thrombus stabilization. Blood 108: 3045–3052.

Dhandapani K, Wade F, Wakade C, Mahesh V, Brann D. 2005. Neuroprotection by stem cell factor in rat cortical neurones involved AKT and NF-κB. J Neurochem 95:9–19.

Foukas L, Daniele N, Ktori C, Anderson K, Jensen J, Shepherd P. 2002. Direct effect of caffeine and theophylline on p110δ and other phosphoinositde 3-kinases. J Biol Chem 277:37124–37130.

Foukas L, Claret M, Pearce W, Okkenhaug K, Meek S, Peskett E, Sancho S, Smith A, Withers J, Vanhaesebroeck B. 2006. Critical role for the p110α phosphoinositide-3-OH kinase in growth and metabolic regulation. Nature 441:366–370.

Furuya F, Hanover J, Cheng S. 2006. Activation of the phosphatidylinositol 3-kinase signaling by a mutant throid hormone β receptor. Proc Natl Acad Sci USA 103:1780–1785.

Furuya F, Guigon C, Zhao L, Lu C, Hanover J, Cheng S. 2007. Nuclear receptor co-repressor (NcoR) is a novel regulator of PI3K signalling. Mol Cell Biol DOI: 10.1128/MCB.00900-07.

Gareus R, Huth M, Breiden B, Nenci A, Rosch N, Haasw I, Bloch W, Sandhoff K, Pasparakis M. 2007. Normal epidermal differentiation but impaired skin-barrier formation upon keratinocyte-restricted IKK1 ablation. Nat Cell Biol 9:461–466.

Gillooly D, Melendez A, Hockaday A, Harnett M, Allen J. 1999. Endoxytosis and vesicular trafficking of immune complexes and activation of phospholipase D by the human high affinity IgG receptor requires distinct phosphoinositides 3-kinase activities. Biochem J 344: 605–611.

Gocek E, Kielbinski M, Marcinkowska E. 2007. Activation of intracellular signalling pathways is necessary for an increase in VDR expression and its nuclear translocation. FEBS Lett 581:1751–1757.

Gomez-Mouton C, Lacalle R, Mira E, Jiminez-Baranda S, Barber D, Carrera A, Martinez A, Manes S. 2004. Dynamic redistribution of raft domains as an organizing platform for signalling during cell chemotaxis. J Cell Biol 164:759–768.

Greger J, Fursov N, Cooch N, McLarney S, Freedman L, Edwards D, Cheskis B. 2007. Phosphorylation of MNAR promotes estrogen activation of PI3 kinase. Mol Cell Biol 27:1904–1913.

Guzman M, Neering S, Upchurch D, Grimes B, Howard D, Rizzieri D, Luger S, Jordan C. 2001. Nuclear factor-κB is constitutively activated in primitive human acute myelogenous leukaemia cells. Blood 98:2301–2307.

Hawkins P, Anderson K, Davidson K, Stephens L. 2006. Signalling through Class I PI3Ks in mammalian cells. Biochem Soc Trans 34:647–662.

Hayakawa M, Kaizawa H, Moritomo H, Koizumi T, Ohishi T, Okada M, Ohta M, Tsukamoto S, Parker P, Workman P, Waterfield M. 2006. Synthesis and biological evaluation of 4-morpholino-2-phenylquinazolines and related

derivatives as novel PI3 kinas e p110α inhibitors. Bioorg Med Chem 14:6847–6858.

Hehner S, Hofmann T, Droge W, Schmitz M. 1999. The anti-inflammatory seaquiterpene lactone parthenolide inhibits NF-κB by targeting the IκB kinase complex. J Immunol 163:5617–5623.

Herdick M, Steinmeyer A, Carlberg C. 2000 Antagonistic action of 25-carboxcylic ester analogue of 1α,25-dihydroxyvitamin D_3 is mediated by a lack of ligand induced vitamin D interaction with coactivators. J Biol Chem 275: 16506–16512.

Hiromura M, Okada F, Obata T, Auguin D, Shibata T, Roumestand C, Noguchi M. 2004. Inhibition of Akt kinase activity by a peptide spanning the βA strand of the proto-oncogene T CL1. J Biol Chem 279:53407–53418.

Hmama Z, Sendide K, Talal A, Garcia R, Dobos K, Reiner N. 2004. Quantitative analysis of phagolysosome fusion in intact cells: Inhibition by mycobacterial lipoarabinomannan and rescue by an 1α,25-dihydroxyvitamin D_3-phosphoinositide 3-kinase pathway. J Cell Sci 117: 2131–2140.

Hu Y, Qiao L, Wang S, Rong S, Meuillet E, Berggren M, Gallegos A, Powis G, Kozikowski A. 2000. 3-(Hydroxymethyl)-bearing phosphatidylinositol ether lipid analogues and carbonate surrogates block PI3-K, Akt and cancer cell growth. J Med Chem 43:3045–3051.

Hughes P, Brown G. 2006. 1α,25-dihydroxyvitamin D_3-mediated stimulation of steroid sulfatase activity in myeloid leukaemic cell lines requires VDR_{nuc}-mediated activation of the RAS/RAF/ERK-MAP kinase signalling pathway. J Cell Biochem 98:590–617.

Hughes P, Twist L, Durham J, Choudhry A, Drayson M, Michell R, Kirk C, Brown G. 2001. Up-regulation of estrone sulfatase activity during retinoid- and vitamin D_3-induced differentiation of HL60 cells. Biochem J 355: 361–371.

Hughes P, Steinmeyer A, Norman A, Chandraratna R, Brown G. 2005. 1α,25-dihydroxyvitamin D_3 stimulates steroid sulfatase activity via a nuclear receptor in acute myeloid leukaemia cell lines but via a membrane receptor in NB4 acute promyelocytic leukaemia cell lines. J Cell Biochem 94:1175–1189.

Huhtakangas J, Olivera C, Bishop J, Zanello L, Norman A. 2004. The vitamin D receptor is present in caveolae-enriched plasma membranes and binds 1α,25$(OH)_2$-vitamin D_3 in vivo and in vitro. Mol Endocrinol 18: 2660–2671.

Ihle N, Paine-Murrieta G, Berggren M, Baker A, Tate W, Wipf P, Abraham R, Kirkpatrick D, Powis G. 2005. The phosphatidylinositol 3-kinase inhibitor PX-866 overcomes resistance to the epidermal growth factor receptor inhibitor gefintib in A-549 human non-small cell lung cancer xenografts. Mol Caner Ther 4:1349–1357.

Jackson S, Scoenwaelder S, Goncalves I, Nesbitt W, Yan C, Wright C, Kenche V, Anderson K, Dopheide S, Yuan Y, Sturgeon S, Prabaharan H, Thompson P, Smith G, Sheperd P, Daniele N, Kulkarni S, Abbott B, Saylik D, Jones C, Lu L, Guiliano S, Hughan S, Angus J, Robertson A, Salem H. 2005. PI 3-kinase p110β: A new target for anti-thrombotic therapy. Nat Med 11:507–514.

Jimenez Del Rio M, Velez-Pardo C. 2006. Insulin-like growth factor-1 prevents Aβ[25-35]/(H_2O_2)-induced apoptosis in lymhocytes by reciprocal NF-κB activation and p53 inhibition via PI3K-dependent pathway. Growth Factors 24:67–78.

Jones G, Strugnell S, DeLuca H. 1998. Current understanding of the molecular actions of vitamin D. Physiol Rev 78:1193–1231.

Keifer J, Guttridge D, Ashburner B, Baldwin A. 2001. Inhibition of NF-κB activity by thalidomide through suppression of IκB kinase activity. J Biol Chem 276: 22382–22387.

Khanal R, Nemere I. 2007. The ERp57/GRp58/1,25D_3-MARRS receptor: Multiple functional roles in diverse cell systems. Curr Med Chem 14:1087–1093.

Kim Y, MacDonald P, Dedhar S, Hruska K. 1996. Association of 1α,25-dihydroxyvitamin D_3-occupied vitamin D receptors with cellular membrane acceptance sites. Endocrinol 137:3649–3658.

Kim S, Kanno A, Ozawa T, Tao H, Umezawa Y. 2007. Nongenomic activity of ligands in the association of androgen receptor with Src. ACS Chem Biol DOI: 10.1021/cb7000439.

Kitawaki J. 2006. Adenomyosis: The pathophysiology of an oestrogen-dependent disease. Best Pract Res Clin Obstet Gynaecol 20:493–502.

Kivineva M, Blauer M, Syvala H, Tammela T, Tuohimaa P. 1998. localization of 1,25-dihydroxyvitamin D3 receptor (VDR) expression in human prostate. J Ster Biochem Mol Biol 66:121–127.

Klopot A, Hance K, Peleg S, Barsony J, Fleet J. 2006. Nucleocytoplasmic cycling of the vitamin D receptor in the enterocyte-like cell line, Caco-2. J Cell Biochem 100:6127–6628.

Kobari M, Yang Z, Gong D, Heissmeyer V, Zhu H, Jung Y, Gakidis M, Rao A, Sekine T, Ikegami F, Yuan C, Yuan J. 2004. Wedelolactone suppresses LPS-induced caspase-11 expression by directly inhibiting the IKK complex. Cell Death Differ 11:123–130.

Kozikowski A, Sun H, Brognard J, Dennis P. 2003. Novel PI analogues selectively block activation of the pro-survival serine, threonine kinase Akt. J Am Chem Sci 125:1144–1145.

Le Mellay V, Lasmoles F, Lieberherr M. 1999. Gα(q/11) and Gβγ proteins and membrane signalling of calcitrol and estradiol. J Cell Biochem 75:138–146.

Lee J, Hmama Z, Mui A, Reiner N. 2004. Stable gene silencing in human monocytic cell lines using lentiviral-delivered siRNA: Silencing of the p110α isoform of phosphoinositide 3-kinase reveals differential regulation of adherence induced by 1α,25-dihydroxyvitamin D3 and bacterial lipopolysaccharide. J Biol Chem 279:9379–9388.

Lee Y, Shim H, Yu H, Song E, Park J, Kwon K, Parl J, Rho H, Park B, Han M, Kim J. 2005. Dimethylsulphoxide induces expression of the tumour suppressor protein PTEN through nuclear factor-κB activation in HL60 cells. Leuk Res 29:401–405.

Lin YZ, Veach R, Togerson T, Hawiger J. 1995. Inhibition of nuclear translocation of transcription factor NF-κB by a synthetic peptide containing a cell membrane-permeable motif and nuclear localization sequence. J Biol Chem 270: 14255–14258.

Liu Q, Ning W, Dantzer R, Freund G, Kelley K. 1998. Activation of protein kinase C-ζ and phosphatidylinositol 3′-kinase and promotion of macrophage differentiation by insulin-like growth factor-1. J Immunol 160:1393–1401.

Lord J, Bunce C, Duncan R, Phillips I, Shennan K, Docherty K, Brown G. 1988. Changes in insulin receptor expression in HL60 cells induced to differentiate towards neutrophils and monocytes. J Mol Endocrinol 1:197–201.

Manning B, Cantley L. 2007. AKT/PKB signalling: Navigating downstream. Cell 129:1261–1274.

Martelli A, Nyakern M, Tabellini G, Bortul R, Tazzari P, Evangelisti C, Cocco L. 2006. Phosphoinositide 3-kinase/Akt signalling pathway and its therapeutic implications for human acute myeloid leukaemia. Leukaemia 20:911–928.

Masiá S, Alvarez S, De Lera A, Darettino D. 2007. Rapid, non-genomic actions of retinoic acid on phosphatidylinositol-3-kinase signalling pathway mediated by the retinoic acid receptor. Mol Endocrinol DOI: 10.1210/me.2007-0062.

Matkovic K, Brugnoli F, Bertagnolo V, Banfic H, Visnjic D. 2006. The role of the nuclear Akt activation and Akt inhibitors in all-trans-retinoic acid-differentiated HL-60 cells. Leukemia 20:941–951.

Melendez A, Harnett M, Allen J. 1999. FcgRI activation of phospholipase Cγ1 and protein kinase C in dibutyrl cAMP-differentiated U937 cells is dependent solely on the tyrosine-kinase activated form of phosphatidylinositol-3-kinase. Immunology 98:1–8.

Monks N, Pardee A. 2006. Targeting the NF-κB pathway in estrogen receptor negative MDA-MB-231 breast cancer cells using small inhibitory RNAs. J Cell Biochem 98: 221–233.

Nakshatri H, Bhat-Nakshatri P, Martin D, Goulet R, Sledge G. 1997. Constitutive activation of NF-κB during progression of breast cancer to hormone-independent growth. Mol Cell Biol 17:3629–3639.

Ng J, Nesaretham K, Reimann K, Lai L. 2000. Effect of retinoic acid and palm oil carotenoids on oestrone sulfatase and oestradiol-17β-hydroxysteroid dehydrogenase activities in MCF-7 and MDA-MB-231 breast cancer cell lines. Int J Cancer 88:135–138.

Norman A. 2006. Vitamin D receptor (VDR): New assignments for an already busy receptor. Endocrinology 147: 5448–5542.

Norman A, Mizwicki M, Norman D. 2004. Steroid hormone rapid actions, membrane receptors and a conformation ensemble model. Nat Rev Drug Discov 3:27–41.

Ociai E, Miura D, Eguchi H, Ohara S, Takenouchi K, Azuma Y, Kamimura T, Norman A, Ishizuka S. 2005. Molecular mechanisms of the vitamin D antagonistic actions of (23*S*)-25-dehydro-1α-hydroyvitamin D_3-23,26-lactone depends on the primary structure of the carboxy-terminal region of the vitamin D receptor. Mol Endocrinol 19:1147–1157.

Ozes O, Mayo L, Gustin J, Pfeiffer S, Pfeiffer L, Donner D. 1999. NF-κB activation by tumour necrosis factor requires Akt serine-threonine kinase. Nature 401:82–85.

Perez-Tenorio G, Alkhor L, Olsson B, Waltersson M, Nordenskjold B, Rutqvist L, Skoog L, Stal O. 2007. PIK3CA mutations and PTEN loss correlate with similar prognostic factors and are not mutually exclusive in breast cancer. Clin Canc Res 13:3577–3584.

Pomel V, Klicic J, Covini D, Church D, Shaw J, Troulin K, Burgat-Charvillon F, Valognes D, Camps M, Chabert C, Gillieron C, Francon B, Perrin D, Leroy D, Gretener D, Nichols A, Vitte P, Carboni S, Rommel C, Schwarz M, Ruckle T. 2006. Furan-2-ylmethylene thiazolidinediones as novel, potent, and selective inhibitors of phosphoinositide 3-kinase γ. J Med Chem 49:3857–3871.

Purohit A, Reed M. 1992. Oestreogen sulfatase activity in hormone-dependent and hormone-independent breast cancer cells: Modulation by steroidal and non-steroidal therapeutic agents. Int J Cancer 50:901–905.

Reed M, Purohit A, Woo L, Newman S, Potter B. 2005. Steroid sulfatase: Molecular biology, regulation, and inhibition. Endocr Rev 26:171–202.

Sasano H, Suzuki T, Nakate T, Moriya T. 2006. New development in intracrinology of breast cancer. Breast cancer 13:129–136.

Selcer K, Hedge P, Li P-K. 1997. Inhibition of estrone sulfatase and proliferation of human breast cancer cells by non-steroidal (*p*-*O*-sulfamoyl)-N-alkanoyl tyramines. Cancer Res 57:702–707.

Selcer K, DiFrancesca H, Chandra A, Li P-K. 2007. Immunohistochemical analysis of steroid sulfatase in human tissues. J Ster Biochem Mol Biol DOI: 10.1016/j.jsbmb.2006.12.105.

Seveau S, Eddy R, Maxfield F, Pierini M. 2001. Cytoskeleton-dependent membrane domain segregation during neutrophil polarization. Mol Biol Cell 12:3550–3562.

Simoncini T, Hafezi-Moghadam A, Brazil D, Ley K, Chin W, Liao J. 2000. Interaction of oestrogen receptor with the regulatory subunit of phosphatidylinositol 3-kinase. Nature 407:538–541.

Sliva D, Rizzo M, English D. 2002. Phosphatditylinositol 3-kinase and NF-κB regulate motility of invasive MDA-MB-231 human breast cancer cells by secretion of the urokinase-type plasminogen activator. J Biol Chem 277:3150–3157.

Sly L, Lopez M, Nauseef W, Reiner N. 2001. 1α,25-dihydroxyvitamin D_3-induced monocyte antimycobacterial activity is regulated by phosphatidylinositol 3-kinase and mediated by the NADPH-dependent phagocyte oxidase. J Biol Chem 276:35482–35493.

Smuc T, Rupreht R, Šinkovec J, Adamski J, Rižner T. 2005. Expression analysis of estrogen-metabolizing enzymes in human endometrial cancer. Mol Cell Endocrinol 248: 114–117.

Songyang Z, Shoelson S, Chaudhuri M, Gish G, Pawson T, Haser W, King F, Roberts T, Ratnofsky S, Lechleider R, Neel B, Birge R, Fajardo E, Chou M, Hanafusa H, Schaffhausen B, Cantley L. 1993. SH2 domains recognize specific phosphopeptide sequences. Cell 72:767–778.

Stanway S, Purohit A, Woo L, Sufi S, Vigushin D, Ward R, Wilson R, Stanczyk F, Dobbs N, Kulinskaya E, Elliott M, Potter B, Reed M, Coombes R. 2006. Phase I study of STX 64 (667 coumate) in breast cancer patients: The first study of a steroid sulfatase inhibitor. Clin Cancer Res 12:1585–1592.

Sun M, Yang L, Feldman R, Sun X-M, Bhalla K, Jove R, Nicosia S, Cheng J. 2003. Activation of phosphatidylinositol 3-kinase/Akt pathway by androgen through interaction of p85α, androgen receptor and Src. J Biol Chem 278:42992–43000.

Suzuki T, Miki Y, Nakamura Y, Moriya T, Ito K, Ohuchi N, Sasano H. 2005. Sex steroid-producing enzymes in human breast cancer. Endocrine-related Cancer 12: 701–720.

Tanaka K, Kubushiro K, Iwamori Y, Okiri Y, Kiguchi K, Ishiwata I, Tsukazaki K, Nozawa S, Iwamori M. 2003. Estrogen sulfotransferase and sulphatase: Roles in the

regulation of estrogen activity in human uterine endometrial carciniomas. Canc Sci 94:871–876.

Tse A, Wan C-K, Shen X-L, Zhu G-Y, Cheung H-Y, Yang M, Fong W-F. 2007. 1,25-dihydroxyvitamin D_3 induces biphasic NF-κB responses during HL-60 leukaemia cells differentiation through protein induction and PI3K/Akt-dependent phosphorylation/ degradation of IκB. Exp Cell Res 313:1722–1739.

Tsunoda Y, Shimizu Y, Tsunoda A, Takimoto M, Sakamoto M, Kusano M. 2006. Steroid sulfatase in breast carcinoma and change in serum estrogen levels after operation. Acta Oncol 45:584–589.

Upadhyay A, Singh S, Chhipa R, Vijayakumar M, Ajay A, Bhat M. 2006. Methyl-β-cyclodextrin enhances the susceptibility of human breast cancer cells to carboplatin and 5-fluorouracil: Involvement of Akt, NF-κB and Bcl-2 toxicol. Appl Pharmacol 216:177–185.

Utsunomiya H, Ito K, Suzuki T, Kitamura T, Kaneko C, Nakata T, Niikura H, Okamura K, Yaegashi N, Sasano H. 2004. Steroid sulfatase and estrogen sulfotransferase in human endometrial carcinoma. Clin Cancer Res 10: 5850–5856.

Van Keymeulen A, Wong K, Knight Z, Govaerts C, Hahn K, Shokat K, Bourne H. 2006. To stabilize neutrophil polarity, PIP_3 and Cdc42 augment RhoA activity at the back as well as signals at the front. J Cell Biol 174:437–445.

Vertino A, Bula C, Chen J, Almeida M, Han L, Bellido T, Kousteni S, Norman A, Manolagas S. 2005. Non-genotropic anti-apoptotic signalling of $1\alpha,25(OH)_2$-vitamin D_3 and analogs through the ligand binding domain of the vitamin D receptor in osteoblasts and osteocytes. Mediation by Src, phosphatidylinositol 3- and JNK kinases. J Biol Chem 280:14130–14137.

Wang T, Tavera-Mendoza L, Laperriere D, Libby E, Macleod N, Nagai Y, Bourdeau V, Konstorum A, Lallement B, Zhang R, Matler S, White J. 2006. Large-scale in silico and microarray-based identification of direct 1,25-dihydroxyvitamin D_3 target genes. Mol Endocrinol 19: 2685–2695.

Wu-Wong R, Nakane M, Ma J, Dixon D, Gagne G. 2006. Vitamin D receptor (VDR) localization in human promyelocytic leukaemia cells. Leuk Lymphoma 47:727–732.

Yanaihara A, Otsuka Y, Iwasaki S, Okai T, Yanaihara T. 2005. Strong expression of steroid sulfatase in human cumulus cells in patients with endometriosis. Fertil Steril 84:465–467.

Ye B, Akamatsu M, Shoelson S, Wolf G, Giorgetti-Peraldi S, Yan X, Roller P, Burke T. 1995. L-O-(2-malonyl)tyrosine: A new phosphotyrosyl mimetic for the preparation of Src homology 2 domain inhibitory peptides. J Med Chem 28: 4270–4275.

Zhang Y, Zhang J, Studzinski G. 2006. AKT pathway is activated by 1,25-dihydroxyvitamin D_3 and participates in its anti-apoptotic effect and cell cycle control in differentiating HL60 cells. Cell Cycle 5:447–451.

Zhou Y, Eppenberger-Castori S, Marx C, Yau C, Scott G, Eppenberger U, Benz C. 2005. Activation of nuclear factor-κB (NFκB) identifies a high-risk subset of hormone dependent breast cancers. Int J Biochem Cell Biol 37: 1130–1144.