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1α,25-Dihydroxyvitamin D_3-Mediated Stimulation of Steroid Sulphatase Activity in Myeloid Leukaemic Cell Lines Requires VDR_{nuc}-Mediated Activation of the RAS/RAF/ERK-MAP Kinase Signaling Pathway

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Abstract 1α,25-dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$) stimulates the activity of steroid sulphatase (STS) in myeloid cells [Hughes et al., 2001, 2005]. This was attenuated by inhibitors of phospholipase D (PLD) (*n*-butanol, 2,3-diphosphoglyceric acid, C_2-ceramide) and phosphatidate phosphohydrolase (PAP) (propranolol and chlorpromazine), but was unaffected by inhibitors of phospholipase C. The 1α,25$(OH)_2D_3$-induced STS activity was also attenuated by inhibitors of protein kinase Cα and protein kinase Cδ (Go 6976, HBDDE and rottlerin), but not by an inhibitor of protein kinase Cβ (LY379196). Additionally, 1α,25$(OH)_2D_3$-induced STS activity was attenuated by inhibitors of RAS (manumycin A), RAF (GW5074), MEK (PD098059 and U1026) and JNK (SP600125), but not p38 (PD169316). 1α,25$(OH)_2D_3$ produced a rapid and long lasting stimulation of the ERK-MAP kinase signalling cascade in HL60 myeloid leukaemic cells. This 'non-genomic' effect of 1α,25$(OH)_2D_3$ blocked by pharmacological antagonists of nuclear vitamin D receptors (VDR_{nuc}) and does not appear to require hetero-dimerisation with the retinoid-X receptor (RXR). Inhibitors of the Src tyrosine kinase (PP1), RAS (manumycin A), RAS–RAF interactions (sulindac sulphide and RAS inhibitory peptide), RAF (GW5074 or chloroquine), and protein kinase Cα (HBDDE) abrogated the 1α,25$(OH)_2D_3$-stimulated increase in ERK-MAP kinase activity. Taken together, these results show that 1α,25$(OH)_2D_3$/VDR_{nuc} activation of the RAS/RAF/ERK-MAP kinase signalling pathway plays an important role in augmenting STS activity in human myeloid leukaemic cell lines. J. Cell. Biochem. 98: 590–617, 2006.

Key words: vitamin D3; steroid sulphatase; genomic and non-genomic signalling; myeloid cells

Inactive steroid sulphates, such as estrone sulphate (E_1S) and dehydroepiandrosterone sulphate (DHEAS), are converted to bioactive estrogens and androgens by the enzyme steroid sulphatase (STS, EC 3.1.6.2) in peripheral tissues [Hanson et al., 2004; Reed et al., 2005]. The expression/activity of STS is physiologically regulated. For example, STS expression/activity rises in discrete areas of the brain [Compagnone et al., 1997] and the skin [Hanley et al., 1997] during the last third of gestation and then falls after birth. STS expression increases in human fallopian epithelial cells during the early luteal phase and recedes during the follicular phase of the menstrual cycle [Yanaihara et al., 2001]. STS activity/expression is also transiently increased during acute or chronic exercise [Riechman et al., 2004] and in the adrenals of stressed animals [Dominguez et al., 1975]. STS activity becomes deregulated as part of several hormone-mediated disorders. A persistent increase in STS expression/activity has observed in blood vessels of spontaneously hypertensive animals [Snyder et al., 2000], in atherosclerotic plaques [Nakamura et al., 2005]

Abbreviations used: 1α,25$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; AML, Acute myeloid leukaemia; E_1S, estrone sulphate; EC_{50}, concentration required to produce a half maximal response; FBS, foetal bovine serum; RXR, retinoid-X receptor; STS, steroid sulphatase; VDR_{nuc}, classical nuclear vitamin D receptor; VDR_{mem}, plasma membrane-associated vitamin D receptor.

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and in skin disorders such as androgenic alopecia, ichthyosis vulgaris, hirsutism, and acne [Chen et al., 2002]. An elevation of STS activity/expression is an indicator of poor outcome in hormone-dependent cancers, particularly those of the breast [reviewed in Nussbaumer and Billich, 2004; Reed et al., 2005].

The factors that regulate the expression/activity of STS are poorly understood [Reed et al., 2005]. However, we have recently shown that 1α,25-dihydroxyvitamin D$_3$ (1α,25(OH)$_2$D$_3$)-drives an increase in STS activity in myeloid leukaemic cell lines [Hughes et al., 2001, 2005].

1α,25(OH)$_2$D$_3$, the active metabolite of vitamin D, regulates mineral homeostasis and also the growth and differentiation of many cell types [Jones et al., 1998; Holick, 2003; Dusso et al., 2005; Nagpal et al., 2005]. 1α,25(OH)$_2$D$_3$ modulates gene expression (genomic responses) via the classical nuclear vitamin D receptor-retinoid X receptor (VDR$_{nuc}$/RXR) heterodimer. This heterodimer functions as a transcription factor, and mediates biological effects that take hours or days to become apparent. Recently, it has been shown that 1α,25(OH)$_2$D$_3$ can also initiate biological effects that occur within seconds to minutes. These 'rapid actions' are mediated by the activation of non-genomic signalling pathways. Moreover, efficient VDR$_{nuc}$/RXR-mediated transactivation of some genes requires co-ordinated activation of both the slow genomic and rapid non-genomic signalling pathways by 1α,25(OH)$_2$D$_3$ liganded VDR$_{nuc}$ (e.g. the rat and human *CYP24*, Dwivedi et al., 2002; Barletta et al., 2004; Nguyen et al., 2004; Nutchey et al., 2005) and human *CYP3A4* [Hara et al., 2002]. A mixture of slow and rapid signals is likely to underlie most of the complex biological effects that are mediated by 1α,25(OH)$_2$D$_3$ [Farach-Carson and Davis, 2003; Fleet, 2004; Boland et al., 2005: Nagpal et al., 2005].

The myeloid leukaemic cell lines HL60, NB4, U937 and THP-1 express VDR$_{nuc}$ [Manfredini et al., 1999], and differentiate into monocyte-like cells in response to physiological concentrations of 1α,25(OH)$_2$D$_3$ [Grande et al., 2002]. Nuclear localisation of VDR$_{nuc}$ [Humeniuk-Polaczek and Marinkowska, 2004], and the generation of VDR$_{nuc}$-driven genomic signals, is absolutely required for monocytic differentiation of HL60, U937 and THP-1 cells [Ji et al., 2002; Ji and Studzinski, 2004]. In contrast, monocytic differentiation of the NB4 acute promyelocytic is not dependent on VDR$_{nuc}$-derived genomic signals, but is initiated by rapid non-genomic signals that, in turn, generate prolonged genomic signals that are required for differentiation [Bhatia et al., 1995; Miura et al., 1999; Hughes et al., 2005]. Hence, HL60 cells, in particular, have been used a models for the studying the mechanisms/consequences of 1α,25(OH)$_2$D$_3$-stimulated VDR$_{nuc}$-mediated direct genomic signalling, whilst NB4 cells have provided a model system for studying VDR$_{nuc}$-independent rapid activation of non-genomic signalling pathways and subsequent indirect genomic signalling events [Miura et al., 1999].

However, recent studies question whether this strict classification of the myeloid cell lines still holds true. For example, 1α,25(OH)$_2$D$_3$ increases the expression and activity of phospholipase D (PLD) and hence the generation of the bioactive diacylglycerol (DAG) in HL60, U937, THP-1 and NB4 cells, and inhibition of PLD activation blocks monocytic differentiation of these cell types [El Marjou et al., 2000]. Similarly, 1α,25(OH)$_2$D$_3$-stimulated translocation and activation of several DAG/calcium-dependent protein kinase C isoforms (PKCα, β and δ) in both NB4 cells [Berry et al., 1996] and HL60 cells [Macfarlane and Menzel, 1994; Pan et al., 1997; Slosberg et al., 2000] is essential for the monocytic differentiation of both cell types. The necessity for the activation of the ERK-MAP kinase pathway in 1α,25(OH)$_2$D$_3$-stimulated monocytic differentiation of NB4 cells was described almost a decade ago [Song et al., 1998], but several studies also show that activation of the ERK-MAP kinase is essential for the 1α,25(OH)$_2$D$_3$-stimulated monocytic differentiation of HL60 cells as well [Marcinkowska et al., 1997; Wang and Studzinski, 2001a,b; Ji et al., 2002]. In fact, it has recently been shown that 1α,25(OH)$_2$D$_3$, acting via VDR$_{nuc}$, increases the activity of RAF, a key activator of the ERK-MAP kinase pathway, in HL60 cells [Studzinski et al., 2005]. Furthermore, activation of the JNK-MAP kinase [Ji et al., 2002] and the p38-MAP kinase pathways [Ji et al., 2002] are required for 1α,25(OH)$_2$D$_3$-driven monocytic differentiation of HL60 myeloid leukaemic cells. Finally, 1α,25(OH)$_2$D$_3$-stimulated, VDR$_{nuc}$-mediated activation of the phosphatidylinositol 3-kinase-Akt-p70 S6 kinase pathway is essential for the differentiation of THP-1 myeloid leukaemic cell lines [Hmama

et al., 1999]. Therefore, in both HL60 and THP-1 cells the biological effects of $1\alpha,25(OH)_2D_3$ are mediated by a mixture of direct slow genomic signals and rapid non-genomic signals.

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 RXR antagonist VTP195393 was a gift from Dr. Roshantha Chandraratna (Vitae Pharmaceuticals, Irvine, CA). All radiolabelled compounds were purchased from Perkin-Elmer Life Science Products-UK Ltd., (Cambridge, UK). The RAF kinase inhibitor GW5074, the MEK kinase inhibitors PD98059, U1026 (and the inactive analogue U1024), the PLD inhibitors 1-butanol (and the inactive *tert*-butanol), 2,3-diphosphoglycerate, the DAG analogue dideca-noylglycerol (DiC10), the PKCδ activator bistratene A, the phosphatidate phosphohydrolase (PAP) inhibitors propranolol and chlorpromazine, the phosphoinositide-specific phospholipase C (PI-PLC) inhibitor U73122 (and the inactive analogue U73344), the PI-PLC activator 2,4,6-trimethyl-*N*-(meta-3-trifluoromethyl-phenyl)-benzenesulfonamide (*m*-3M3FBS) and the src tyrosine kinase family inhibitor PP1 were purchased from the Sigma Chemical Co. (Poole Dorset, UK). The PLD inhibitor C2-ceramide (and the inactive analogue dihydro-C2-ceramide), the phosphatidylcholine-specific phospholipase C (PC-PLC) inhibitor D-609, the PKCα activator thymeleatoxin, the PKCβ inhibitor LY379196, the JNK inhibitor SP600125 and the p38 MAP kinase inhibitor, PD169316 were purchased from Calbiochem (Nottingham, England, UK). The protein kinase C inhibitors GF 109293X, Gö 6976, HBBDE and rottlerrin were purchased from Affiniti Research products (Exeter, UK).

Accurate definition of the non-genomic signalling pathways involved in $1\alpha,25(OH)_2D_3$-stimulation of STS activity in myeloid leukaemic cells is reliant on the use of selective chemical inhibitors. Our choice of inhibitor combinations and incubation times were guided by previous studies performed in HL60 and other myeloid cell lines, and in which the pharmacological specificities of the inhibitors have been previously characterised [Bertagnolo et al., 1999, 2004; Cambien et al., 1999; Yen et al., 1999; El Marjou et al., 2000; Slosberg et al., 2000; Lopez-Pedrera et al., 2001, 2004; Wang and Studzinski, 2001a,b; Lewandowski et al., 2002; Neri et al., 2002; Martelli et al., 2003; Wang et al., 2003; Zhao et al., 2004; Fong et al., 2005; Glasow et al., 2005; Lal et al., 2005]. We have followed the 'guidelines' outlined in Davies et al. [2000] for the evaluation of the effects of pharmacological inhibitors on cellular processes. To this end, we determined, in parallel experiments, that the effect of the inhibitors on the $1\alpha,25(OH)_2D_3$-stimulated increase in STS activity occurred at concentrations that blocked activation of an authentic physiological substrate of the protein kinase as measured using the same cell extract (data not shown). Secondly, where possible, the effects of two structurally unrelated inhibitors of the intracellular target under investigation have been examined.

To ensure that any inhibition of $1\alpha,25(OH)_2D_3$-stimulated increase in STS activity produced by the pharmacological inhibitors was not related to non-specific toxicity, we determined the effects of all the compounds on cell viability. Release of lactate dehydrogenase (LDH) was measured at the start and end of the incubation periods of cells with each of the inhibitors to ensure that the agents had not caused a decrease in cell viability. LDH was assayed using a colorimetric LDH assay kit (Sigma) as described in the manufacturers protocol. Inhibitors were deemed to have had a toxic effect, and observations were discarded, if the percentage LDH release had either significantly increased during the time course of the experiment or was greater than 20% of the total cellular LDH.

Cell Culture

The ML-1, HL60, NB4, U937 and THP-1 cell lines were grown in RPMI 1640 medium supplemented with 10% foetal calf serum (FBS), penicillin (100 U/ml) and streptomycin (100 μg/ml) in 95% air/5% CO_2 at 37°C. KG-1 and KG-1a cells were grown in RPMI 1640 medium supplemented with 20% FBS and antibiotics. Cells were seeded at 2.5×10^5 cells per ml as 10 ml cultures in 25 cm^2 flasks. Differentiation was induced with 0.1–100 nM

1α,25(OH)$_2$D$_3$ for 3 days, unless otherwise stated.

Antisense MAP Kinase Oligonucleotide Treatment of Myeloid Cell Lines

Cell cultures were treated for 24 h with 5 μM of a completely phosphorothioate-modified antisense oligodeoxynucleotide (ERK1/2-AS-ODN, 5′-GCCGCCGCCGCCGCAT-3′, Biomol Research Laboratories, Inc., Essex, UK) directed against the initiation codon and the subsequent 14 bases of the mouse/human p42 ERK2 mRNA or with a phosphorothioate modified oligonucleotide with the same base composition as the ERK2 antisense oligonucleotide, but with a randomly scrambled sequence (R-ODN, 5′-CGCGCGCTCGCGCACCC-3′). After treatment cell cultures were washed twice in pre-warmed tissue culture medium, resuspended and stimulated with 100 nM 1α,25(OH)$_2$D$_3$ either for 60 min (ERK-MAP kinase assay) or 72 h (STS assay).

Depletion of Plasma-Membrane Cholesterol

HL60, U937 and THP-1 cells were adapted to growth in RPMI 1640 supplemented containing 2% FBS for 2 weeks prior to the start of the experiment. The cholesterol sequestering agent methyl-β-cyclodextrin (MCD) was used to disrupt cholesterol-rich microdomain formation (CMF). Cells were treated with 10 mM MCD for 2 h prior to stimulation with 100 nM 1α,25(OH)$_2$D$_3$.

Measurement of Steroid Sulphatase Activity in Cell Fractions

Cells were collected by centrifugation and resuspended 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 resuspended in 0.5 ml of buffer by sonication and its protein concentration was measured (Bio-Rad kit, Hemel Hempstead, Herts, UK). STS activity was assayed at 37°C in 0.02 M Tris-HCl (pH 7.5) containing 20 μM E$_1$S (spiked with ~100,000 dpm of [^{3}H]-E$_1$S) in a final volume of 100 μl. Reactions were started with 30–80 of μg protein and were quenched after 60 min with 900 μl of ice-cold 0.1M sodium bicarbonate containing 5000 dpm of [^{14}C]-E$_1$ to determine the recovery of the extraction process. Nine hundred fifty microliters of the resulting mixture were extracted with 3 ml of toluene and the organic and aqueous layers were separated. Eight milliliters 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.

Measurement of Inositol Phosphate Production in Intact HL60 Cells

HL60 cells were labelled with 10 μCi/ml myo-[2-^{3}H]inositol in inositol-free RPMI 1640 supplemented with antibiotics and 10% dialysed FBS (MP Biomedicals, London, UK) for 96 h. Cells were then harvested by centrifugation, washed twice with inositol-free RPMI 1640 medium containing 0.5% FBS, 20 mM HEPES at pH 7.2, 20 mM LiCl, and bovine serum albumin (BSA, 1 mg/ml) and then resuspended at a density of 2×10^7 cells/ml and incubated at 37°C for a further 60 min. A portion (0.1 ml) of the cell suspension was transferred to a microcentrifuge tube and incubated at 37°C for 15 min. Reactions were initiated by adding 100 nM 1α,25(OH)$_2$D$_3$, 10 μM histamine or 100 μM m-3M3FBS, and were terminated after 30 min by adding 200 μl of ice-cold 10% perchloric acid. This mixture was neutralized with 1.2 M KOH containing 10g/L phytic acid. After 30 min in an ice bath, the tubes were centrifuged and the supernatants were diluted fivefold with distilled water and applied to 1 ml Dowex AG 1-X8 (formate) anion exchange columns (Bio-Rad Life Sciences Ltd.). Free inositol was washed through the column with 5 ml of distilled water, and inositol glycerophosphates by 10 ml of 60 mM ammonium formate containing 5 mM sodium tetraborate. Total inositol phosphates were eluted with 10 ml of 1.2 M ammonium formate and 0.1 M formic acid. The radioactivity of the [^{3}H]inositol phosphates was determined by liquid scintillation spectrometry.

Intracellular Calcium Measurements

A change in intracellular Ca^{2+} concentration was monitored by using the Ca^{2+}-sensitive fluorescent dye Fura-2. Exponentially growing HL60 cells were harvested and washed three times in HEPES-buffered saline; 1.5 μM $CaCl_2$ was added to the final wash. The cells were

resuspended at a final concentration of 10^6/ml. Cells were treated with Fura-2/AM (5 μM) and 0.012% pluronic F127 in the dark for 40 min at room temperature. Cells were washed three times in HEPES buffered saline to remove unincorporated FURA-2/AM. The cells were then incubated in the dark at room temperature for a further 30 min, so that the incorporated dye was completely de-esterified. One milliliters samples of the cells were treated with 100 nM 1α,25$(OH)_2D_3$, 10 μM histamine, or 100 μM m-3M3FBS. Changes in the fluorescence ratio were measured at an emission wavelength of 500 nm, and dual excitation wavelengths of 340 nm and 380 nm. The calibration of the fluorescence ratio versus $[Ca^{2+}]_i$ was performed as described by Grynkiewicz et al. [1985].

Measurement of Phospholipase D Activity in Intact Cells

Myeloid cells were treated with 100 nM 1α,25$(OH)_2D_3$ for 72 h and then washed three times with RPMI 1640 containing 10% FBS. After a further 2 h at 37°C, the cells were harvested by centrifugation into HEPES buffered saline (25 mM HEPES, pH 7.4, 125 mM NaCl, 0.7 mM $MgCl_2$, 0.5 mM EGTA, 10 mM glucose, 1 mg/ml BSA) and resuspended in the same buffer containing 10 μCi/ml [^{3}H]-oleic acid for 3 h. Excess oleic acid was removed by repeated washing in HEPES buffered saline. Cells were resuspended (10^7/ml) in HEPES buffered saline containing 2% ethanol (v/v) and incubated at 37°C for a further 60 min. PLD activity was assessed in 100 μl aliquots as described below. To assess whether 1α,25$(OH)_2D_3$ directly stimulates PLD activity, myeloid cells grown in RPMI 1640/10% FBS were labelled for 20–24 h with 2.5 μCi/ml [^{3}H]-oleic acid. Cells were washed three times in HEPES buffered saline (composition as above), and resuspended in the same buffer at a concentration of 2×10^6 cells/ml. Five hundred microliters aliquots of cells were then exposed to 100 nM 1α,25$(OH)_2D_3$ in the presence of 0.5% ethanol for up to 300 min at 37°C.

Reactions were quenched by addition of 2.5 ml of chloroform/methanol (2/1, v/v). Lipids were Folch extracted and aliquots were taken to measure total [^{3}H]-labelled lipids. The remainder of the sample was dried down under a stream of nitrogen, resuspended in the mobile phase and spotted on silica gel G plates. The plates were developed using a solvent system consisting of ethyl acetate–isooctane–acetic acid–water 13:2:3:10 (v/v). This solvent system allowed separation of phosphatidic acid (PA, $R_f = 0.24$), phosphatidylethanol (PEt, $R_f = 0.34$), neutral lipids ($R_f = 0.95–1.0$) and phospholipids ($R_f = 0$). The product of the trans-phosphatidylation reaction phosphatidyethanol (PtdsEtOH) was identified by co-migration with an authentic standard. The spot corresponding to [^{3}H]-PtdsEtOH was scraped from the TLC plate and quantitated by liquid scintillation spectrometry. Results are expressed as the amount of ^{3}H-PtdsEtOH formed as a percentage of the total amount of ^{3}H-labeled phospholipid in each experiment. [^{3}H]cpm comigrating with PtdsEtOH were determined for each set of samples in the absence of ethanol, and these background counts were subtracted from each data point.

Measurement of In vivo ERK1/2 MAP Kinase Activity in Myeloid Cells

1α,25$(OH)_2D_3$-stimulated ERK1/2 activity was measured using the FACE™ ERK1/2 chemiluminescent Elisa assay kit (Actif Motif Europe, Rixensart, Belgium). The assay measures both total ERK1/2 and phosphorylated ERK1/2 levels using sensitive primary antibodies and an HRP-conjugated secondary antibody. This assay is more sensitive and quantitative than normal Western blotting approaches for the measurement of ERK-MAP kinase activity [Bulayeva et al., 2004].

Myeloid cells were harvested by centrifugation and resuspended (1.5 ml) at a concentration of $\sim 1 \times 10^6$ cells per ml in pre-warmed RPMI 1640 containing 2% FBS and antibiotics in 24-well tissue culture dishes. After a 24 h equilibration period, the cell suspension was stimulated with 100 nM 1α,25$(OH)_2D_3$ for up to 72 h. At the required times duplicate, 0.5 ml sample were taken for the determination of phosphorylated ERK-MAP kinase (*p*ERK) and total ERK-MAP kinase activity. Each sample was centrifuged at 30,000*g* for 60 s at 4°C to terminate the reaction. The supernatant was aspirated and the cell pellets was immediately fixed with 100 μl of 8% (v/v) formaldehyde in phosphate buffered saline for 30 minutes 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 LP4 tube and chemiluminescence was measured in a Berthold LB 953 luminometer. As the reactions were terminated a parallel 0.5 ml cell sample was labelled with crystal violet as described in the kit manufacturers instructions for 30 min. The cells were lysed by sonication and absorbance measured at 595 nM. This was done to ensure that equal numbers of cells were assayed and to normalise data from the ERK-MAPK assay.

Statistical Analysis

The statistical significance between groups of data was analysed by the Students *t*-test or where appropriate by the Mann–Whitney rank sum or Kruskal–Wallis one way analysis of variance tests using either the Minitab^{TM} 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 Steroid Sulphatase Activity in Myeloid Leukaemic Cell Lines

1α,25(OH)$_2$D$_3$ stimulates the activity and expression of STS in HL60 and NB4 acute myeloid leukaemia (AML) cell lines and upregulation of the ability to desulphate steroids is a marker of myeloid cell differentiation [Hughes et al., 2001, 2005]. The basal level of STS was observed to increase in parallel with an increased maturation status of cell lines. Figure 1a shows that the basal STS activity in extracts of promyeloid HL60 cell (FAB-M2) was 0.43 ± 0.09 nmoles E_1 formed per hour per mg of protein ($n = 482$), as compared to 1.11 ± 0.05 in extracts from monoblastic THP-1 cells (FAB-M5) ($n = 122$, $P < 0.00001$). This difference is consistent with the notion that an increase in STS activity is linked to myeloid cell maturation.

1α,25(OH)$_2$D$_3$ failed to increase STS activity in the immature KG-1a (■) and KG-1 (●) cell lines (Fig. 1b). These cell lines typify the least differentiated type of AML. 1α,25(OH)$_2$D$_3$ also failed to upregulate STS activity in freshly isolated blast cells from FAB-M0 leukaemia patients (data not shown) and in the K562 immature myelo-erythroblastic cell line (data not shown). In contrast, 1α,25(OH)$_2$D$_3$ stimulated STS activity in myeloid cell lines representative of the FAB-M2 to M5 sub-classifications. The concentration of 1α,25(OH)$_2$D$_3$ required to produce a half-maximal increase in sulphatase activity (EC_{50}) in ML-1 (○, early M2) cells was 7.5 ± 1.3 nM ($n = 3$), and the Hill slope of the dose response curve was not significantly different from unity (0.97 ± 0.1, Fig. 1b). In HL60 cells (△, FAB-M2), the 1α,25(OH)$_2$D$_3$ concentration-response curve was best fitted by a sigmoidal curve with a Hill coefficient not significantly different from unity ($N_H = 0.90 \pm 0.18$, $n = 12$, $r^2 = 0.93$), and the EC_{50} was 10.2 ± 1.2 nM. Similarly, EC_{50}'s obtained for THP-1 (◇, M5 subtype) and the histiocytic U937 (□, M5 subtype) cell lines were 6.2 ± 1.1 nM ($n = 8$) and 10.3 ± 2.9 nM ($n = 3$), respectively, and again the Hill slopes were 0.84 ± 0.1 and 1.1 ± 0.29, respectively. One hundred nanomolar 1α,25(OH)$_2$D$_3$ also increased STS activity in the AML-M3 cell line NB4 (▽, EC_{50} was 0.77 ± 0.4 nM, $n = 5$). However, the Hill slope for the 1α,25(OH)$_2$D$_3$ dose response curve in NB4 cells was much steeper being 2.87 ± 0.2 ($n = 5$).

Figure 1c,d show the effects of the VDR_{nuc} antagonists ZK159222 and ZK168281 [Toell et al., 2001; Carlberg, 2003] on the 1α,25(OH)$_2$D$_3$-stimulated increase in STS in the acute myeloid leukaemic cell lines. Both antagonists prevented the increase in STS activity produced by 100 nM 1α,25(OH)$_2$D$_3$ in HL60, THP-1 and U937 cells in a dose responsive fashion. The concentrations of ZK159222 that inhibited the increase in STS activity produced by 100 nM 1α,25(OH)$_2$D$_3$ by 50% (IC_{50}) in HL60, THP-1 and U937 cells were 705 ± 89 nM (Fig. 1c, $n = 4$), 1190 ± 230 nM (Fig. 1c, $n = 4$) and 964 ± 165 nM (Fig. 1c, $n = 4$), respectively. Similarly, IC_{50} values obtained using HL60, THP-1 and U937 cells were 512 ± 41 nM (Fig. 1d, $n = 4$), 278 ± 39 nM (Fig. 1d, $n = 4$) and 288 ± 112 nM (Fig. 1d, $n = 4$), respectively. In contrast, ZK159222 and ZK168281 failed to have any significant effect on the increase in STS activity produced when NB4 cells were stimulated with 100 nM 1α,25(OH)$_2$D$_3$ (Fig. 1c,d).

1α,25-Dihydroxyvitamin D$_3$ Does Not Stimulate Phosphoinositide-Specific Phospholipase C or Mobilise Intracellular Calcium in HL60 Cells

1α,25(OH)$_2$D$_3$ can activate several PI-PLC isoforms leading to the generation of the second

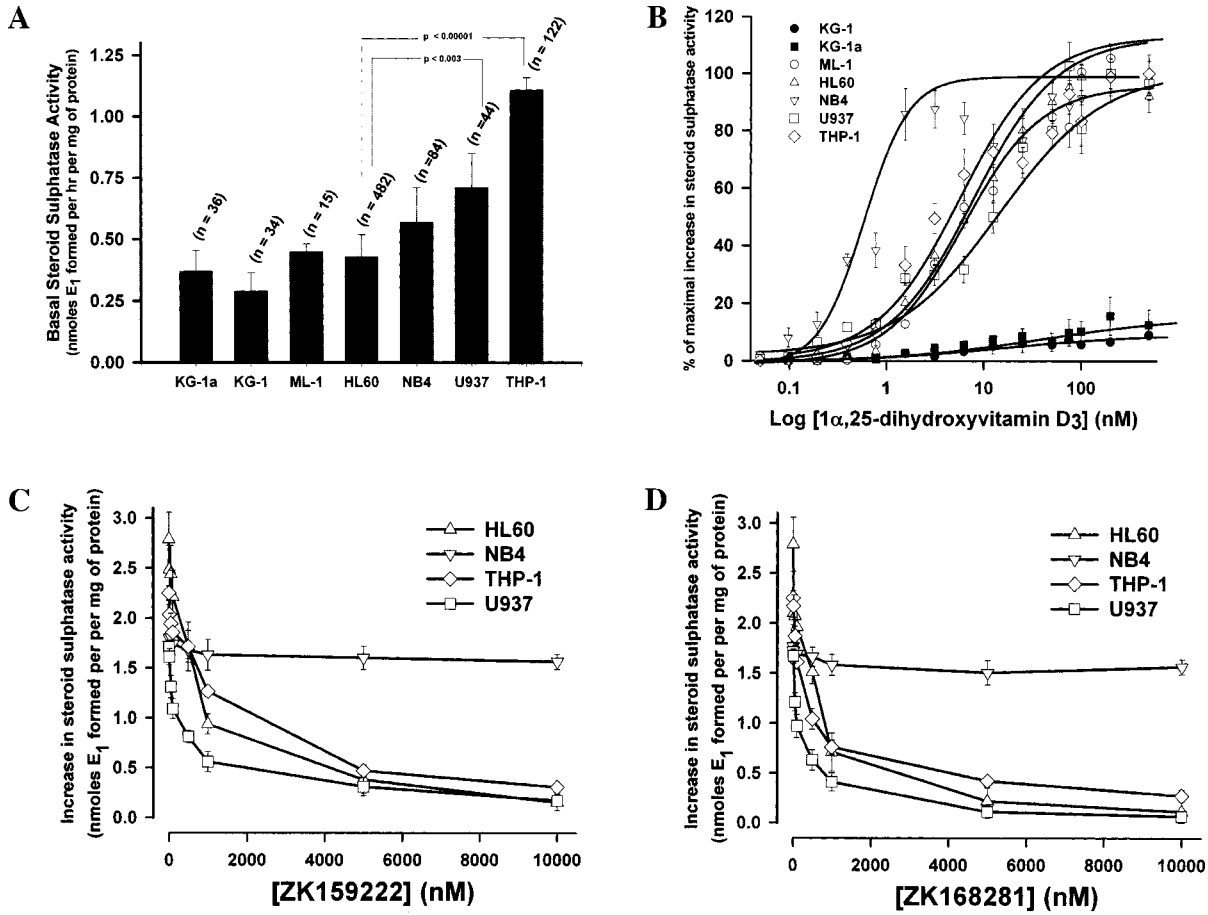

Fig. 1. $1\alpha,25(OH)_2D_3$ stimulates steroid sulphatase (STS) activity in myeloid leukaemic cell lines. **Panel A** shows the basal STS activity in KG-1a (n = 38), KG-1 (n = 34), ML-1 (n = 15), HL60 (n = 482), NB4 (n = 84), U937 (n = 44) and THP-1 (n = 122) cell extracts. Cells were harvested as they reached a density of $\sim 1 \times 10^6$ cells per ml. Basal STS activity was measured in cell extracts as described in the Experimental section. All data are expressed as mean ± SEM of the basal STS activity expressed as the nmoles of E_1 formed per hour per mg of protein. **Panel B** shows the increases in STS activity in KG-1a (n = 3, ●), KG-1 (n = 3, ■), ML-1 (n = 3, ○), HL60 (n = 12, △), NB4 (n = 8, ▽), U937 (n = 6, □) and THP-1 (n = 4, ◇) myeloid leukaemic cell lines to stimulated for 72 h with $1\alpha,25(OH)_2D_3$ (0.1–100 nM). Data is expressed as the mean ± SEM of the maximal increase in STS activity over basal. EC_{50}'s were estimated using the 'pharmacology' module of the Sigmaplot™ (version 9.0) graphical software package after fitting the data to a sigmoidal dose response curve. **Panels C and D** shows that ZK159222 and ZK168281 inhibit $1\alpha,25(OH)_2D_3$-stimulated STS activity in myeloid cells. Cells were treated with either ZK159222 or ZK168281 (10–10,000 nM) for 2 h prior to stimulation with 100 nM $1\alpha,25(OH)_2D_3$. Microsomal STS activity was measured 72 h later. Data are expressed as the mean ± SEM of the percentage inhibition of the increase in STS activity stimulated by 100 nM $1\alpha,25(OH)_2D_3$. Data are from four experiments performed in quadruplicate. The inhibition curves were fitted to a sigmoidal dose response curve and the IC_{50}'s were estimated using the 'pharmacology' module of the Sigmaplot™ (version 9.0) graphical software package.

messengers $Ins(1,4,5)P_3$ and DAG in many cell types. Indeed, $1\alpha,25(OH)_2D_3$-stimulated monocytic differentiation of HL60 cells has been associated with a slow but persistent cytoplasmic/nuclear translocation of PI-PLC-β_2 and PI-PLC-β_3 [Neri et al., 1999], which may serve a role in controlling nuclear cytoskeletal organisation [Bertagnolo et al., 2004]. Activation of PI-PLC is characterised by an increase the intracellular levels of inositol phosphates and an associated transient rise in intracellular Ca^{2+} [$[Ca^{2+}]_I$]. We tested whether $1\alpha,25(OH)_2D_3$ can drive PI-PLC activation by measuring total inositol phosphate formation in [^{3}H]-inositol-labelled HL60 cells. Figure 2a shows that 100 nM $1\alpha,25(OH)_2D_3$ did not have any significant effect on inositol phosphate levels in HL60 cells. In contrast, Figure 2a shows that both histamine (10 μM) and the synthetic PI-PLC activator *m*-3M3FBS (100 μM) stimulated PI-PLC activation in HL60 cells. Similar observations have been made in U937 and THP-1 cells [data not

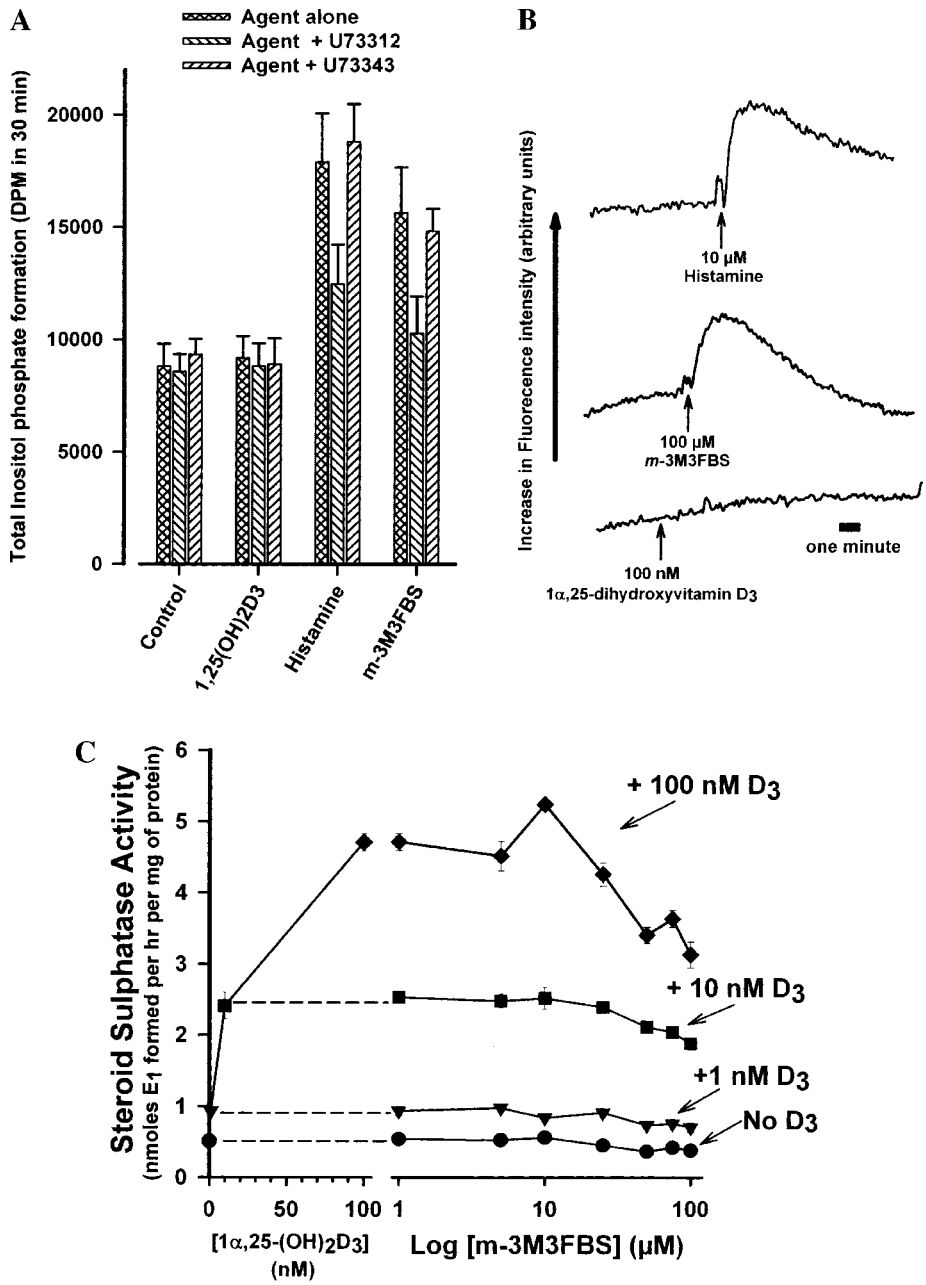

Fig. 2. 1α,25(OH)$_2$D$_3$ does not stimulate phosphoinositide-specific phospholipase C nor does it mobilise intracellular calcium in HL60 cells. **Panel A** shows that 1α,25(OH)$_2$D$_3$ does not stimulate phosphoinositide-specific phospholipase C in [^{3}H]-inositol-labelled HL60 cells. HL60 cells were stimulated with either 100 nM 1α,25(OH)$_2$D$_3$, 10 μM histamine or 100 μM 2,4,6-trimethyl-*N*-(meta-3-trifluoromethyl-phenyl)-benzenesulfonamide (*m*-3M3FBS) in the presence of 20 mM LiCl for 30 min. In some experiments cells were incubated with the phospholipase C inhibitor U-73122 (5 μM) or its inactive analogue U-73343 (5 μM) for 30 min prior to stimulation. [^{3}H]-inositol phosphates were isolated and separated as described in the Experimental section. The results are shown as the mean ± SEM of the total [^{3}H]-inositol phosphates recovered (expressed as DPM per 30 min). The data are from in three experiments performed in triplicate. **Panel B** shows that 1α,25(OH)$_2$D$_3$ does not mobilise intracellular calcium in HL60 cells. HL60 cells were labelled with the fluorescent calcium indicator FURA-2/AM as described in the Experimental section and were stimulated with either 100 nM 1α,25(OH)$_2$D$_3$, 10 μM histamine or *m*-3M3FBS. Changes in $[Ca^{2+}]_i$ were measured as described by Grynkiewicz et al. [1985]. Representative traces are shown. Similar results were obtained in three further experiments. **Panel C** shows that m-3M3FBS alone had no effect on STS activity in HL60 cells neither did it augment the 1α,25(OH)$_2$D$_3$-stimulated increase in STS activity. Cells were stimulated with m-3M3FBS (1–100 μM) alone or in combination with 1α,25(OH)$_2$D$_3$ (1–100 nM) for 72 h. Results are shown as the mean ± SEM of the microsomal STS activity (in nmoles E_1 formed per hour per mg of protein). The results shown were obtained in two separate experiments performed in quadruplicate.

shown; Bae et al., 2003]. In keeping with these observations, 100 nM 1α,25$(OH)_2D_3$ failed to mobilise $[Ca^{2+}]_i$ in HL60 cells. Both histamine and *m*-3M3FBS stimulated a robust mobilisation of $[Ca^{2+}]_i$ (Fig. 2b). Neither histamine (data not shown) nor 1–100 μM *m*-3M3FBS had any effect on either basal STS activity or the increase in STS activity, that is induced by 1–100 nM 1α,25$(OH)_2D_3$ (Fig. 2c). Activation of PI-PLC, and the subsequent elevation of $[Ca^{2+}]_i$, is not a sufficient stimulus for the induction of STS in HL60 cells.

It is perhaps not surprising that the phosphoinositide-specific phospholipase C (PI-PLC) inhibitor U73122 (1–10 μM, see Fig. 2a) and its inactive analogue U73433 had no effect on either basal or 1α,25$(OH)_2D_3$-stimulated increase in STS activity in HL60 cells (data not shown). Similarly, the phosphatidylcholine-specific phospholipase C (PC-PLC) inhibitor D-609 (10–500 μM) failed to have any significant effect on basal STS activity in HL60 cells and the increase induced by 100 nM 1α,25$(OH)_2D_3$ (data not shown).

Gardner et al. [1997] have shown that although 1α,25$(OH)_2D_3$ fails to produce a rapid Ins(1,4,5)P_3-dependent increase in $[Ca^{2+}]_i$ in HL60 cells, the basal $[Ca^{2+}]_i$ rises slowly to 20%–30% above basal after 72–96 h exposure to 1α,25$(OH)_2D_3$. It has been suggested that 1α,25$(OH)_2D_3$ increases Ca^{2+} influx by directly opening store operated calcium channels (SOCC). Vazquez et al. [2000] have suggested that both 1α,25$(OH)_2D_3$-stimulated Ca^{2+}/calmodulin-dependent kinase II are required for the opening of SOCCs. On the other hand, Santillan et al. [2004] have proposed that 1α,25$(OH)_2D_3$-liganded VDR_{nuc} binds to and activates TRPC3, a protein that is involved in SOCC function. To test whether a gradual rise in $[Ca^{2+}]_i$ plays a role in the 1α,25$(OH)_2D_3$-stimulated increase in STS activity in HL60 cells, we used the sarco(endo)plasmic Ca^{2+}-ATPase (SERCA) inhibitor thapsigargin. This agent directly activates SOCC in HL60 cells and produces a slow but persistent increase in $[Ca^{2+}]_i$ similar to that produced by 1α,25$(OH)_2D_3$ [Gardner et al., 1997; Korchak et al., 2001]. Treatment of HL60 cells with 10–100 nM thapsigargin did not have any significant effect on basal STS activity (data not shown) or on the 1α,25$(OH)_2D_3$-stimulated increase in STS activity in HL60 cells (data not shown). The general intracellular Ca^{2+} chelator BAPTA-AM (5 μM), the specific Ins(1,4,5)P_3-receptor blocker aminoethoxydiphenyl borate [2-APB, 100 μM, Lee et al., 2005] and the voltage-dependent calcium channel blockers verapmil [10 μM, Nagashima and Goto, 2000] and nifedipine [25 μM, Feuerstein and Cooper, 1984] also failed to have any effect on the 1α,25$(OH)_2D_3$-mediated stimulation of STS activity in HL60 cells (data not shown).

Taken as a whole these data show that 1α,25$(OH)_2D_3$ does not activate PI-PLC or PC-PLC in HL60 cells. Furthermore, the data suggests that an increase in $[Ca^{2+}]_i$, whether it be from rapid activation of Ins(1,4,5)P_3-sensitive stores or a slower influx from extracellular sources following activation of a gated calcium channel, does not provide a sufficient stimulus to increase STS activity or augment 1α,25$(OH)_2D_3$-stimulated STS activity in HL60 cells.

Inhibition of Phospholipase D Blocks the 1α,25-Dihydroxyvitamin D_3-Stimulated Increase in Steroid Sulphatase Activity in Myeloid Leukaemic Cell Lines

PLD plays a role in a diverse range of cellular processes that include receptor signalling, control of intracellular membrane transport, and reorganization of the actin cytoskeleton [Liscovitch et al., 2000].

The basal PLD activity in HL60 cells (FAB-M3) was $0.038 \pm 0.009\%$ (expressed as the amount of ^{3}H-PtdsEtOH formed as a percentage of total ^{3}H-lipids, $n = 7$), and was $0.37 \pm 0.06\%$ ($n = 5$, $P < 0.001$ vs. HL60) in the relatively more mature THP-1 (FAB-M5) cell line. Figure 3a shows that there is a positive correlation between the mean basal PLD activity and the mean basal STS activity ($r^2 = 0.972 \pm 0.11$, $P < 0.02$), suggesting that PLD activity may play a role in regulating STS activity. Figure 3b shows that basal PLD activity was increased by 1α,25$(OH)_2D_3$ (100 nM) in the ML-1 (3.3 fold), HL60 (9.3 fold), NB4 (sevenfold), U937 (~twofold), and THP-1 (~twofold) cell lines. 1α,25$(OH)_2D_3$ failed to have any effect on basal PLD activity in both KG-1 and KG-1a cells. Therefore, the ability of 1α,25$(OH)_2D_3$ to augment the expression and activity of PLD in myeloid leukaemic cell lines varies with their maturation status with the most immature cells being resistant to induction. In the responsive cell lines, the 1α,25$(OH)_2D_3$-stimulated increase in PLD activity was blocked by pre-treatment

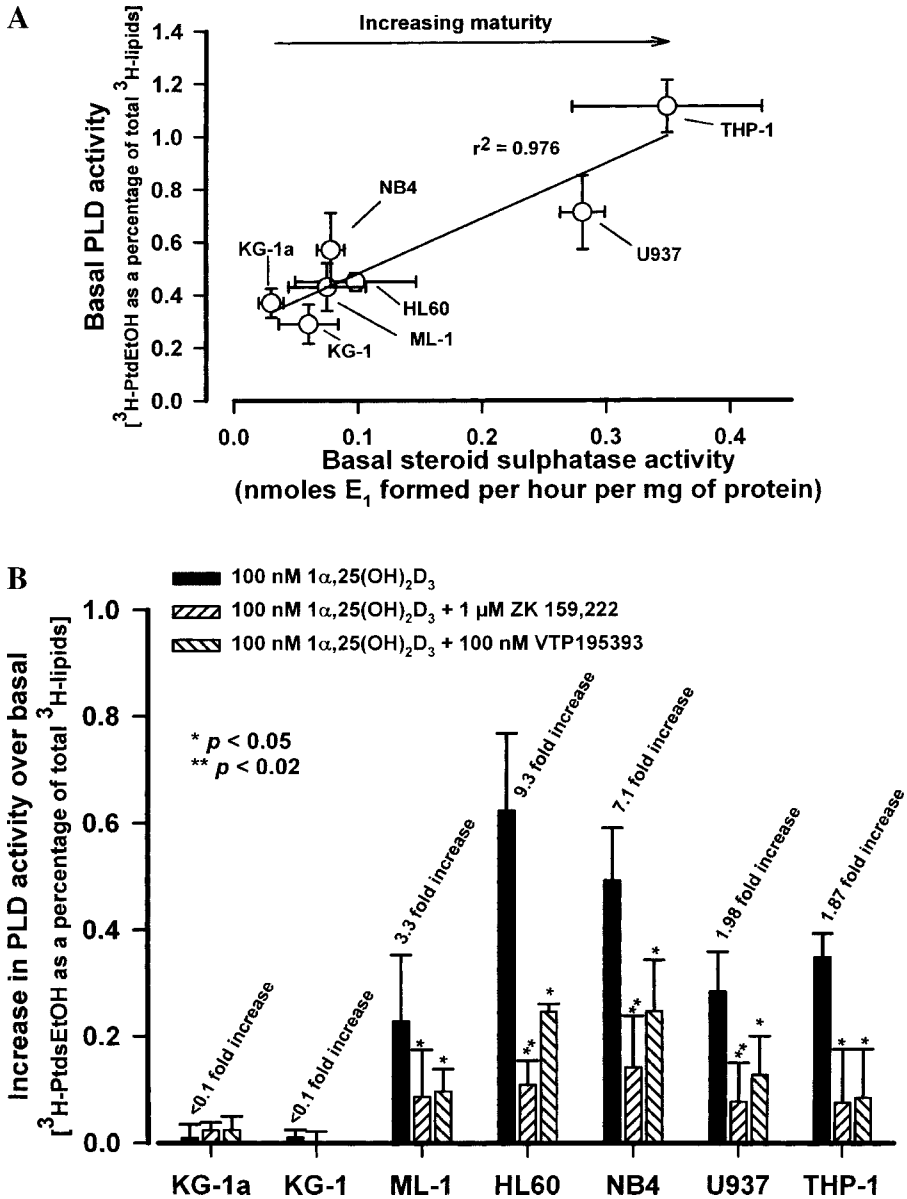

Fig. 3. 1α,25(OH)$_2$D$_3$ increases basal phospholipase D (PLD) activity in myeloid leukaemic cell line. **Panel A** shows a correlation between basal STS activity and the basal level of PLD activity in myeloid leukaemic cell lines. Basal PLD activity was measured as described in the Experimental section. Data is shown as the mean ± SEM of the basal PLD activity. Three separate experiments were performed in triplicate. The basal STS activity was measured as described in the legend to Figure 1a. **Panel B** shows that 1α,25(OH)$_2$D$_3$ stimulates basal PLD activity in ML-1, HL60 NB4, U937 and THP-1 cells but not in KG-1a or KG-1 cells. Cells were stimulated with 100 nM 1α,25(OH)$_2$D$_3$ for 72 h. In some experiments the cells were treated with 1 μM ZK159222 or 100 nM VTP195393 for 2 h prior to stimulation. Basal PLD activity was measured as described in the Experimental section. Data is shown as the mean increase in PLD activity over basal ± SEM and was obtained in three experiments each performed in triplicate.

of the myeloid cells with either the VDR$_{nuc}$ antagonist ZK159222 (1 μM, Fig. 3b) or the RXR antagonist VTP195393 (100 nM, Fig. 3b). These findings suggest that PLD is a direct target for the VDR/RXR genomic signalling system. An increase in the expression of PLD1 and PLD2 protein has previously been associated with both monocytic and granulocytic differentiation of myeloid leukaemic cell lines [El Marjou et al., 2000; Di Fulvio and Gomez-Cambronero, 2005].

An increase in PLD activity is observed within a few minutes of stimulation of human colonocytes [Khare et al., 1999a,b], rat skeletal muscle [Faccinetti et al., 1998] and chick

myoblasts [Morelli et al., 1996] with 1α,25 $(OH)_2D_3$, suggesting the involvement of non-genomic signalling pathways in the activation mechanism. An increase in [^{3}H]-PtdsEtOH accumulation was observed in [^{3}H]-oleic acid labelled HL60 cells within 5 min of stimulation with 100 nM 1α,25$(OH)_2D_3$. [^{3}H]-PtdsEtOH accumulatation reached a peak 30–60 min after stimulation and then slowly declined to close to basal by 36–48 h (Fig. 4a). The rapid 1,25 $(OH)_2D_3$-stimulated increase in PLD activity (measured at 60 min) was dose dependent ($IC_{50} = 7.42 \pm 1.7$ nM) (data not shown). Rapid increases in PLD activity in response to 1α,25$(OH)_2D_3$ were also observed in NB4, U937 and THP-1 cell lines, but not in KG-1 and KG-1a cell lines (Fig. 4b).

Primary alcohols inhibit PLD-mediated effects by diverting phospholipid metabolism away from the production of PA towards a biologically inactive non-metabolizable phosphatidylalcohol [Liscovitch et al., 2000] or by interfering with its association with PKCα [Hu and Exton, 2005]. In preliminary experiments we demonstrated that 0.3% (v/v) *n*-butanol completely blocked the 1α,25$(OH)_2D_3$-stimulated increase in PLD activity, whilst *t*-butanol, a tertiary alcohol that is not a substrate for PLD-mediated transphosphatidylation, failed to have any effect (data not shown). Figure 4c shows that the 1α,25$(OH)_2D_3$-stimulated increase in STS activity was blocked by *n*-butanol in a dose-responsive fashion ($IC_{50} = 0.15 \pm 0.04\%$), whilst the tertiary alcohol *t*-butanol failed to any inhibitory effect. Short-chain membrane permeant ceramides also inhibit the activity of PLD [Mansfield et al., 2004]. Treatment of HL60 cells with 50 μM C_2-ceramide for 2 h prior to the addition of 1α,25$(OH)_2D_3$ reduced the 1α,25$(OH)_2D_3$-mediated increase in STS activity by approximately 60% (data not shown, $P < 0.002$). Dihydro-C_2-ceramide (50 μM), which does not inhibit PLD, failed to have any significant effect (data not shown, $P > 0.6$). Similarly, 2,3-diphosphoglycerate (5 mM), a competitive inhibitor of PLD [Kanaho et al., 1993], also inhibited the 1α,25$(OH)_2D_3$-mediated increase in STS activity by >50% (data not shown).

PLD predominantly hydrolyses phosphatidylcholine to generate the intracellular lipid second messenger PA [reviewed in Liscovitch et al., 2000]. Treatment of HL60 cells with 100 μM PA alone for 72 h did not effect STS activity (data not shown). But, addition of 100 μM PA produced a small but significant left-shift in the 1α,25$(OH)_2D_3$ concentration response curve: the EC_{50} was reduced from 6.3 ± 1.4 nM in the absence of PA to 2.9 ± 1.1 nM in the presence of PA ($n = 3$, $P = 0.03$). PA can also be generated by the diacylglycerol kinase (DGK) catalysed phosphorylation of DAG. R59022 (30 μM), a non-selective DGK inhibitor [Batista et al., 2005], failed to have any significant effect on the 1α,25$(OH)_2D_3$-mediated increase in STS activity (data not shown). Therefore, PA has only a minor, perhaps modulatory, role in the 1α,25$(OH)_2D_3$-mediated increase in STS activity in myeloid leukaemic cells. Phosphatidate phosphohydrolase (PAPH) converts PA into the *bone fide* lipid mediator DAG. The PAPH-mediated degradation of PA can be inhibited by propranolol or chlorpromazine [Rabano et al., 2004]. Both these agents (at 100 μM) inhibited the increase in STS activity produced by stimulation of HL60 cells with 100 nM 1α,25$(OH)_2D_3$ by >80% (Fig. 4d).

Inhibition of Protein Kinase Cα and δ Isoforms Attenuates the 1α,25-Dihydroxyvitamin D_3-Mediated Increase in Steroid Sulphatase Activity in HL60 Cells

The pan-PKC activator 12-*O*-tetradecanoylphorbol-13-acetate (TPA) induces monocytic differentiation of myeloid leukaemic cell lines. Figure 5a shows that TPA (16 nM) failed to have any significant effect on STS activity in HL60 cells, despite causing a marked decrease in cell proliferation, increased cell adhesion and the acquisition of other well characterised markers of monocytic differentiation (CD11b and CD14 expression or acquisition of the ability to reduce NBT, data not shown). Similarly, thymeleatoxin (100 nM), a specific activator of PKCα [Yanagita et al., 2000] did not increase STS activity in HL60 cells (Fig. 5b). The PKCβ-specific activator 12-deoxyphorbol-13-*O*-phenylacetate-20-acetate (Doppa, 100 nM) [Ryves et al., 1994; Pongracz et al., 1996] and the PLCδ-specific activator bistratene A (100 nM) [Griffiths et al., 1996] induce incomplete differentiation of HL60 cells along the monocyte pathway. Neither Doppa nor bistratene A increased STS activity in HL60 cells (Fig. 5b). These results indicate that activation of PKC alone is not a sufficient stimulus to increase the activity of STS.

We, therefore, tested whether TPA had any effect on the increase in STS activity in HL60

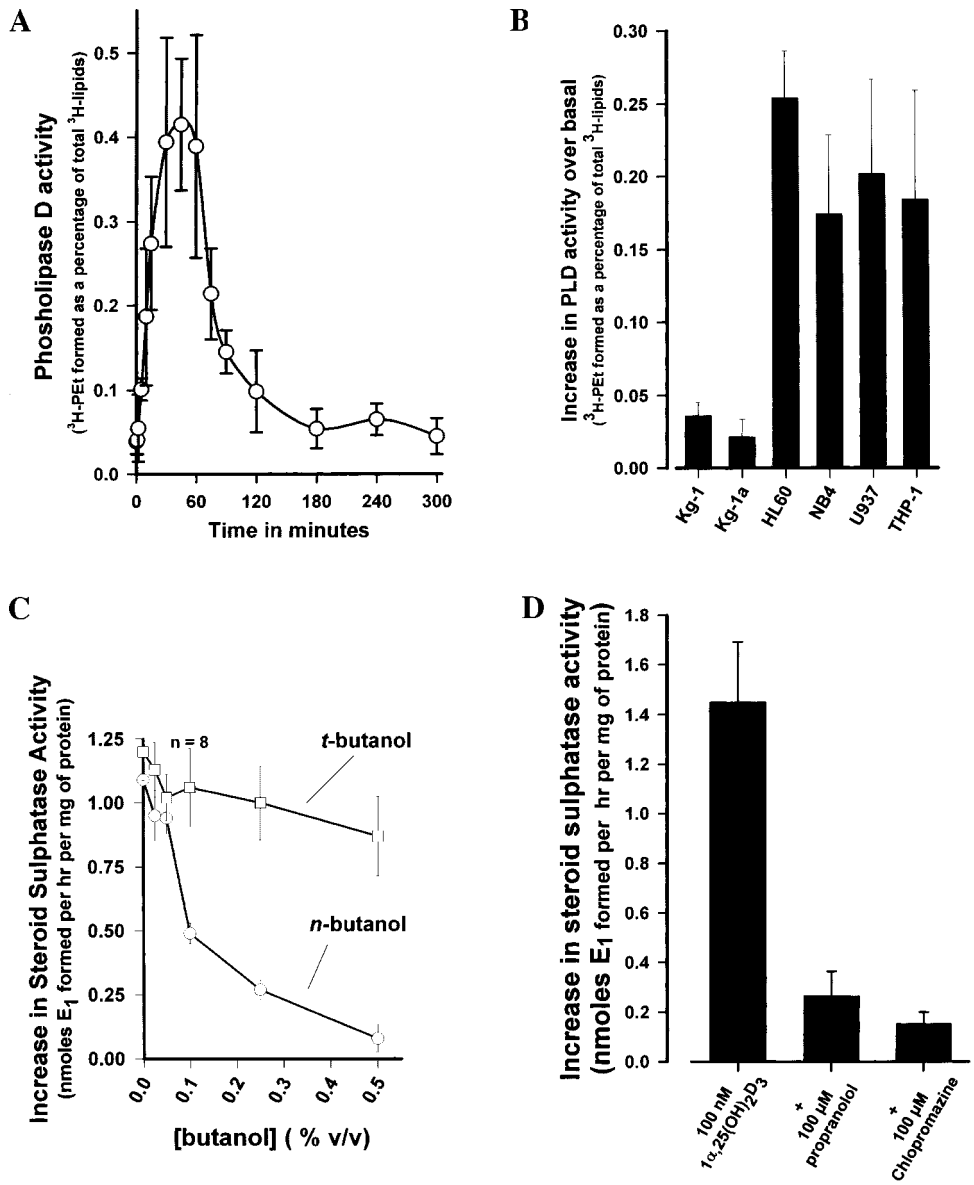

Fig. 4. 1α,25(OH)$_2$D$_3$ stimulates PLD activity in HL60 and other myeloid leukaemic cell lines. **Panel A** shows the time course of PLD activation by 1α,25(OH)$_2$D$_3$ in HL60 cells. Cells were pre-labelled with [^{3}H]-oleic acid as described in the Experimental section. Cells were treated with 100 nM 1α,25(OH)$_2$D$_3$ in the presence of 0.5% (v/v) ethanol and were harvested at various times up to 5 h after stimulation. [^{3}H]-phosphatidylethanol was separated by thin layer chromatography as described in the Experimental section. Results are presented as the amount of [^{3}H]-phosphatidylethanol formed (expressed as a percentage of the total [3H]-labelled lipids) ± SEM and was obtained from three experiments each performed in triplicate. **Panel B** shows that 1α,25(OH)$_2$D$_3$ increased in PLD activity in HL60, NB4, U937 and THP-1 cells but not KG-1 and KG-1a cells. [^{3}H]-oleic acid labelled cells were stimulated with 100 nM 1α,25(OH)$_2$D$_3$ in the presence of 0.5% (v/v) ethanol for 60 min. PLD activity was quantitated as described above. **Panel C** shows that inhibition of PLD with the primary alcohol *n*-butanol blocks the 1α,25(OH)$_2$D$_3$-stimulated increase in STS activity in HL60 cells. Cells were treated with 0.05–0.5% (v/v) *n*-butanol or its inactive analogue *t*-butanol for 2 h prior to stimulation with 100 nM 1α,25(OH)$_2$D$_3$ for 72 h. Results are shown as the mean ± SEM of the increase in microsomal STS activity over basal (in nmoles E$_1$ formed per hour per mg of protein). The results shown were obtained in eight experiments each performed in quadruplicate. **Panel D** shows that inhibition of phosphatidate phosphohydrolase (PAPH) blocks the 1α,25(OH)$_2$D$_3$-stimulated increase in STS activity in HL60 cells. Cells were treated with 100 μM propranolol or chlorpromazine for 2 h prior to stimulation with 100 nM 1α,25(OH)$_2$D$_3$. After 72 h cells were harvested and microsomal extracts prepared as described in the Experimental section. Results (from three experiments performed in quadruplicate) are shown as the mean ± SEM of the increase STS activity over basal (in nmoles E$_1$ formed per hour per mg of protein).

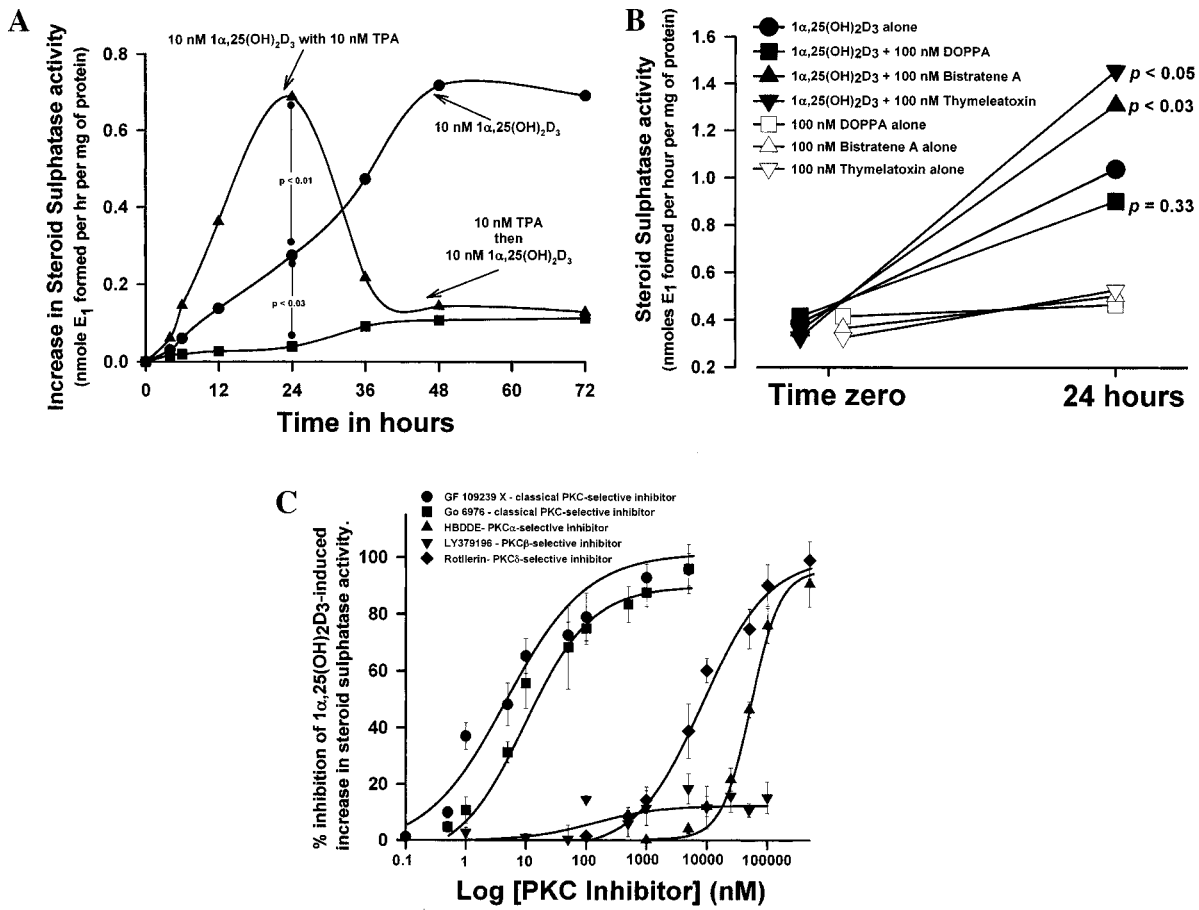

Fig. 5. The role of protein kinase C isoforms in the $1\alpha,25(OH)_2D_3$-mediated increase in STS activity in HL60 cells. **Panel A**: HL60 cells were treated with 100 nM $1\alpha,25(OH)_2D_3$ alone (●), 10 nM phorbol dibutyrate (TPA) for 24 h prior to the addition of 100 nM $1\alpha,25(OH)_2D_3$ (■) or 100 nM $1\alpha,25(OH)_2D_3$ and 10 nM phorbol dibutyrate added at the same time(▲). At various times after stimulation the cells were harvested and STS activity in the microsomal fraction was determined as described in the Experimental section. The results are shown as the mean increase in STS activity (nmoles E_1 formed per hour per mg of protein) over basal and were obtained in three experiments performed in triplicate. **Panel B** shows the effects the PKCα activator thymeleatoxin (▼,▽), the PKCβ activator 12-deoxyphorbol-13-*O*-phenylacetate-20-acetate (■,□, DOPPA) and the PKCδ activator bistratene A (▲,△) added alone (open figures) or in combination (closed figures) with 100 nM $1\alpha,25(OH)_2D_3$ on STS activity in HL60 cells. After 24 hours stimulation the cells were harvested and STS activity in the microsomal fraction was determined as described in the Experimental section. All data points are mean ± SEM of quadruplicate determinations, and each experiment was performed a minimum of four times. **Panel C** shows the effects of inhibitors of protein kinase C on the $1\alpha,25(OH)_2D_3$-stimulated increase in STS activity in HL60 cells. Cells were pre-treated with the pan-PKC inhibitor GF 109293X (●,1–100 nM), the cPKCα/β specific inhibitor Gö 6976 (■, 1–100 nM), the cPKCα-specific inhibitor HBBDE (▲, 1–10 μM), the cPKCβ inhibitor LY379196 (▼, 1–100 nM) or the cPKCδ-, α-elective inhibitor rottlerin (◆, 1–100 μM) for 2 h prior to addition of 100 nM $1\alpha,25(OH)_2D_3$. All data points are mean ± SEM of quadruplicate determinations, and each experiment was performed a minimum of four times.

cells, that is induced by a sub-maximal concentration of $1\alpha,25(OH)_2D_3$. Figure 5a shows that 10 nM $1\alpha,25(OH)_2D_3$ failed to stimulate an increase in STS activity when HL60 cells were treated with TPA (10 nM) for 24 h prior to treatment with the seco-steroid [■]. A downregulation of PKC activity is a consequence of prolonged phorbol ester treatment [Leontieva and Black, 2004]. Hence, the inability of HL60 cells to increase STS activity after prolonged phorbol ester treatment suggests that activation of protein kinase C is required for the $1\alpha,25(OH)_2D_3$-mediated increase in STS activity. Figure 5a shows that when TPA and a sub-maximal dose of $1\alpha,25(OH)_2D_3$ were added to the cultures at the same time [▲], there was an augmentation in STS activity over the first 12–24 h that was followed by a rapid decrease in STS activity. The latter is probably explained by downregulation of PKC activity. Figure 5b shows the effects on STS activity in HL60 cells of co-treatment with a sub-maximal dose of

1α,25(OH)$_2$D$_3$ and thymeleatoxin, Doppa or bistratene A HL60 cells were co-treated for just 24 h to limit toxicity, prevent desensitisation of PKC and to prevent conversion of Doppa into the less specific PKC activator 12-deoxyphorbol-13-*O*-phenylacetate (Dopp) [Ryves et al., 1994]. Co-administration of 100 nM Doppa [■] had little effect, but both 100 nM thymeleatoxin and 100 nM bistratene A [▲] produced small but significant augmentations of the increase in STS activity that is provoked by 10 nM 1α,25(OH)$_2$D$_3$. This data suggest that PKCα and PKCδ, but not PKCβ, play a modulatory role on 1α,25(OH)$_2$D$_3$-stimulated increase in STS activity in HL60 cells.

Figure 5c shows data from experiments in which HL60 cells were treated with inhibitors of various PKC isoforms for 2 h prior to treatment with 100 nM 1α,25(OH)$_2$D$_3$. Low nanomolar concentrations of GF 109293X inhibit several PKC isoforms (relative potency = $\alpha > \beta I > \varepsilon > \delta > \zeta$). The 1α,25(OH)$_2D_3$-stimulated increase in STS activity in HL60 cells was blocked by GF 109293X [●] in a dose responsive fashion with an IC_{50} of 4.1 ± 2.02 nM ($n = 6$). Nanomolar concentrations of Gö 6976 have been reported to selectively inhibit Ca^{2+}/DAG-dependent classical PKC isoenzymes (PKCα and PKCβI), whilst having no effect on the Ca^{2+}-independent PKC isoenzymes (PKCδ,ε and ζ). Gö 6976 [■] inhibited 1α,25(OH)$_2$D$_3$-stimulated increase in STS activity in HL60 cells in a dose-responsive fashion with an IC_{50} of 9.2 ± 1.6 nM ($n = 4$). 2,2′,3,3′,4,4′-Hexahydroxy-1,1′-biphenyl-6,6′-dimethanol dimethyl ether (HBDDE) is a relatively selective inhibitor of PKCα and γ ($IC_{50} \approx 50$ μM) that does not affect βI and βII or δ isoforms. The 1α,25(OH)$_2$D$_3$-stimulated increase in STS activity in HL60 cells was blocked by HBDDE [▲] at concentrations ($IC_{50} = 49.9 \pm 5.7$μM, $n = 3$) compatible with its inhibitory effects on PKCα. In contrast, the PKCβ-selective inhibitor LY379196 (▼) [Slosberg et al., 2000] failed to have any significant effect on the 1α,25(OH)$_2$D$_3$-stimulated increase in STS activity in HL60 cells even at concentrations 10–100 fold higher than that required to block TPA-mediated differentiation of HL60 cells [Slosberg et al., 2000]. Finally, Figure 5c shows that rottlerin, a selective PKCδ inhibitor ($IC_{50} = 3$–6 μM), inhibited the 1α,25(OH)$_2$D$_3$-stimulated increase in STS activity in HL60 cells ($IC_{50} = 8.2 \pm 1.7$ μM). Rottlerin was effective at concentrations that inhibit PKCδ and below those that inhibit other PKC isoforms. Therefore, PKCδ is a component in the 1α,25 (OH)$_2$D$_3$-stimulated increase in STS activity in HL60 cells.

1α,25-Dihydroxyvitamin D$_3$-Stimulates ERK-MAP Kinase Activity in HL60, NB4, U937 and THP-1 Cells but not in KG-1 and KG-1a Cells

Several workers have suggested that activation of the ERK-MAP kinase signalling pathway plays an important role in 1α,25(OH)$_2$D$_3$-mediated differentiation of myeloid leukaemic cell lines. Figure 6a shows that basal ERK-MAP kinase activity was lowest in resting KG-1a and KG-1 cell lines and highest in unstimulated THP-1 cells, providing a tentative link between maturation status of the myeloid leukaemic cell lines and ERK-MAP kinase activation. Furthermore, Figure 6a demonstrates a positive correlation between the mean basal ERK-MAP kinase activity and the mean basal STS activity ($r^2 = 0.901 \pm 0.02$, $P < 0.035$), which suggests a role for ERK-MAP kinases in regulating basal STS activity.

In the experiments shown in Figure 6b, HL60 cells were treated with 100 nM 1α,25(OH)$_2$D$_3$ for 72 h, and total ERK1/2 and *p*ERK were measured by a chemiluminescent ELISA assay. The levels of total ERK1/2 remained fairly constant over the 72-h time course of the experiment (Fig. 6b, □). Thus, 1α,25(OH)$_2$D$_3$-VDR signalling system does not increase ERK1/2 expression in HL60 cells. Figure 6b also shows the time course of ERK-MAP kinase activation (measured as an increase in *p*ERK) in HL60 cells stimulated with 100 nM 1α,25(OH)$_2$D$_3$. *p*ERK content was increased within 15–60 min of stimulation and rose steadily to reach a plateau at 16–20 h after which *p*ERK content gradually returned to basal within 36–48 h. Wang and Studzinski [2001] have reported a similar time course for 1α,25(OH)$_2$D$_3$-mediated activation of ERK-MAP kinase in HL60 cells. The time course of the 1α,25(OH)$_2$D$_3$-stimulated increase in ERK-MAP kinase activity in HL60 cells was not significantly altered by treating cells with actinomycin D and cycloheximide (data not shown). Hence, synthesis of new protein is not required. 1α,25(OH)$_2$D$_3$ (100 nM) produced an ~fivefold increase in the relative abundance of *p*ERK in HL60 and NB4 cells, and an ~twofold increase in *p*ERK content was seen in U937 and THP-1 cells (Fig. 6c). However, 1α,25(OH)$_2$D$_3$ failed to stimulate ERK-MAP kinase activity in KG-1 and KG-1a cells (Fig. 6c).

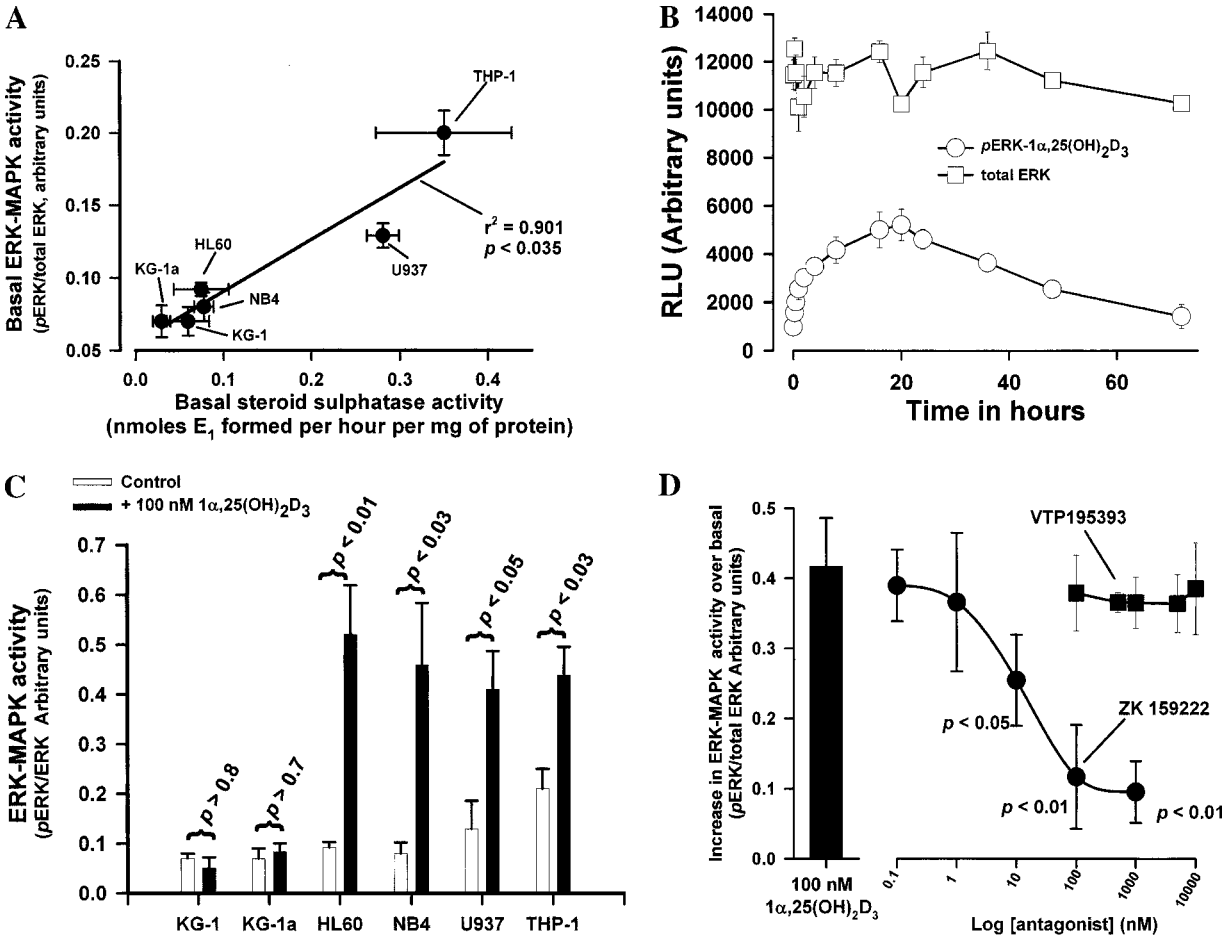

Fig. 6. $1\alpha,25(OH)_2D_3$-stimulates ERK-MAP kinase activity in HL60, NB4, U937 and THP-1 cells but not in KG-1 and KG-1a cells. **Panel A** shows there is a correlation between basal ERK-MAP kinase activity and basal STS activity in myeloid leukaemic cell lines. Basal ERK-MAP kinase activity cell was measured as described in the Experimental section. Total ERK and phosphorylated ERK-MAP kinase (*p*ERK) content was measured using the Actif Motif FACE™ ERK1/2 chemiluminescent Elisa assay kit following the manufacturers instructions. Data is expressed as the mean ± SEM of the ratio of *p*ERK to total ERK-MAP kinase (in arbitrary units) and were obtained from three experiments performed in triplicate. The basal STS activity data is the same as shown in Figure 1a. **Panel B** shows the time-course of the activation of ERK-MAP kinase by $1\alpha,25(OH)_2D_3$ in HL60 cells. Cells were stimulated with 100 nM $1\alpha,25(OH)_2D_3$ and at various times after stimulation aliquots and total ERK (□) and *p*ERK (○) content was measured as described in the Experimental section. The data were obtained in two experiments each performed in triplicate. **Panel C** shows that $1\alpha,25(OH)_2D_3$ stimulates ERK-MAPK activity in HL60, NB4, U937 and THP-1 cells but not in KG-1a or KG-1 cells. ERK-MAP kinase activation was measured 60 min after stimulation with 100 nM $1\alpha,25(OH)_2D_3$ as described in the Experimental section. Data are expressed as the mean ± SEM of the ratio of *p*ERK to total ERK-MAP and were obtained from two experiments each performed in triplicate. **Panel D** shows that $1\alpha,25(OH)_2D_3$-stimulated ERK-MAPK activity in HL60 cells was inhibited by the VDR antagonist ZK159222 (●), but not the RXR antagonist VTP195393 (■). Cells were treated with ZK159222 (0.1–1000 nM) or VTP195393 (100–10,000 nM) for 2 h prior to stimulation with 100 nM $1\alpha,25(OH)_2D_3$. After 60 min the cells were harvested/fixed and total ERK and *p*ERK content measured by chemiluminescent Elisa as described in the Experimental section. The data are expressed as the mean ± SEM of the ratio of *p*ERK to total ERK and were obtained in three experiments performed in triplicate. The inhibition curves were fitted to a sigmoidal dose response curve and the IC_{50}'s were estimated using the 'pharmacology' module of the Sigmaplot™ (version 9.0) graphical software package.

VDR_{nuc} is Involved in the $1\alpha,25$-Dihydroxyvitamin D_3-Stimulated ERK-MAP Kinase Activity in HL60 Cells

The VDR_{nuc} antagonist ZK159222 inhibited the increase in *p*ERK content produced by 100 nM $1\alpha,25(OH)_2D_3$ in HL60 cells in a dose-responsive fashion ($IC_{50} = 197.7 \pm 32.8$ μM, $N_H = -0.92 \pm 0.09$, Fig. 6d). However, it should be noted that the inhibition of $1\alpha,25(OH)_2D_3$-stimulated increase in ERK-MAP kinase activity was incomplete and ~20% of the $1\alpha,25(OH)_2D_3$-stimulated activity was resistant to inhibition by a 100-fold excess of ZK159222.

Figure 6d also shows that the RXR antagonist VTP195393 failed to have any effect on the 1α,25(OH)$_2$D$_3$-stimulated increase in ERK-MAP kinase activity in HL60 cells. Thus, the formation of VDR-RXR heterodimers is not required for the 1α,25(OH)$_2$D$_3$-stimulated increase in ERK-MAP kinase activity in HL60 cells.

A common theme in steroid hormone nuclear receptor-mediated activation of the ERK-MAP kinase signalling cascade is the targeting of the liganded nuclear receptors to cholesterol-rich micro-domains (CMF) in the plasma membrane [Edwards, 2005]. Locating nuclear hormone receptors in CMF allows them to come into contact with the scaffolding/adaptor proteins that couple to and activate multiple signalling pathway [Edwards, 2005]. Recent reports show that significant amounts of VDR$_{nuc}$ are found in CMF in the NB4 myeloid leukaemic cell line [Huhtakangas et al., 2004]. Treatment of HL60, U937 and THP-1 cells with the cholesterol-depleting agent methyl-β-cyclodextrin (MβCD, 10 mM) inhibited 1α,25(OH)$_2$D$_3$-stimulated ERK-MAP kinase activity by ~75% (Fig. 7a) the 1α,25(OH)$_2$D$_3$-stimulated increase in STS activity by >60%. In avian embryonic skeletal muscle cells 1α,25(OH)$_2$D$_3$ induced translocation of tyrosine phosphorylated VDR$_{nuc}$ to the plasma membrane requires an intact microtubular network [Capiati et al., 2002]. Disruption of microtubules with 100 nM colchicine inhibited the 1α,25(OH)$_2$D$_3$-stimulated ERK-MAP kinase activity in HL60, U937 and THP-1 cells by ~70% (Fig. 7a), and the 1α,25(OH)$_2$D$_3$-stimulated increase in STS activity by ~60% (Fig. 7b).

1α,25-Dihydroxyvitamin D$_3$-Stimulated ERK-MAP Kinase Activity in HL60 Cells is Dependent on PKCα, Src Tyrosine Kinase, RAS and RAF

The magnitude and duration of the activation of ERK-MAP kinase is tightly controlled and requires multiple regulatory inputs [Ebisuya et al., 2005]. Activation of the ERK-MAP kinase-signalling pathway is thought to be a downstream element in the PKC signalling in HL60 cells [Marcinkowska et al., 1997]. Down regulation of PKCα expression in HL60 cells, by pretreatment for 24 h with 100 nM TPA, produced a

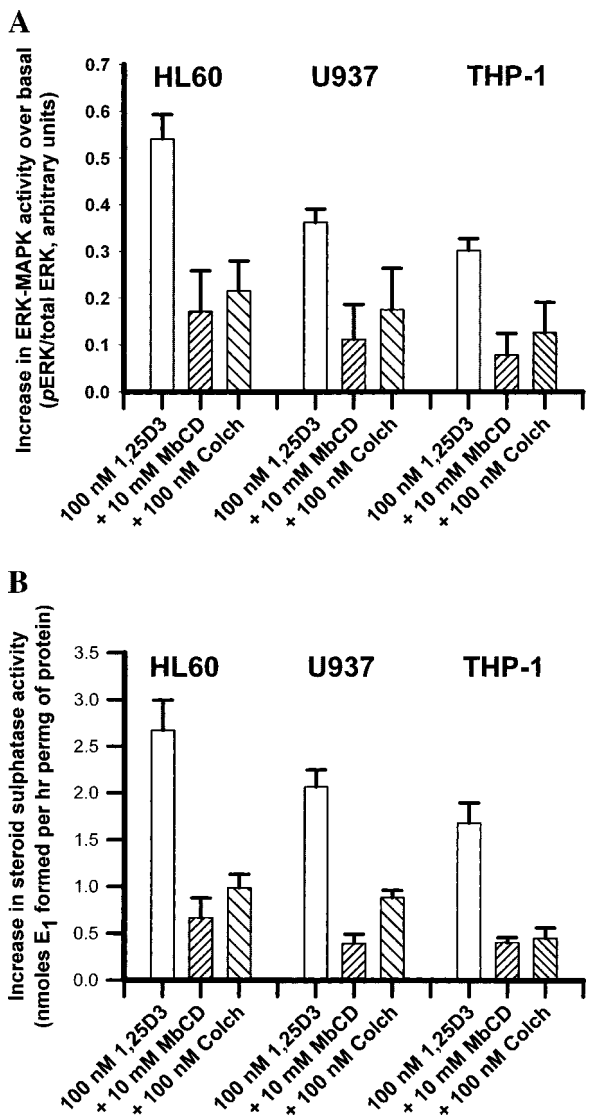

Fig. 7. 1α,25(OH)$_2$D$_3$-stimulated ERK-MAP kinase activity and STS activity in HL60, U937 and THP-1 myeloid leukaemic cells are inhibited by depletion of membrane cholesterol with methyl-β-cyclodextrin (10 mM) or disruption of microtubules with colchicines (100 nM). The cholesterol depletion experiments were performed in HL60, U937 and THP-1 cells that had been conditioned to growth in 2% FBS for 2 weeks prior to experimentation. **Panel A** shows that 1α,25(OH)$_2$D$_3$ stimulated ERK-MAPK activity in HL60, NB4, U937 and THP-1 cells was inhibited by MβCD and colchicine. Cells were treated with 10 mM methyl-β-cyclodextrin or 100 nM colchicine for 2 h prior to stimulation with 100 nM 1α,25(OH)$_2$D$_3$. ERK-MAP kinase activation was measured 60 min after stimulation with 100 nM 1α,25(OH)$_2$D$_3$ as described in the Experimental section. Data are expressed as the mean ± SEM of the ratio of *p*ERK to total ERK-MAP and were obtained from two experiments each performed in triplicate. **Panel B** shows that MβCD and colchicine inhibit the1α,25(OH)$_2$D$_3$-stimulated increase in STS activity in HL60, U937 and THP-1 cells. Cultures were treated with the inhibitors for 2 h prior to stimulation with 100 nM 1α,25(OH)$_2$D$_3$. After 24 h the cells were harvested and microsomal STS activity measured as described in the Experimental section. Data are shown as the mean ± SEM of the inhibition of 1α,25(OH)$_2$D$_3$-stimulated STS activity and were obtained in two experiments each performed in quadruplicate.

75% inhibition ($n = 3$, $P = 0.0021$) in activation of ERK-MAP kinase that is stimulated with 100 nM $1\alpha,25(OH)_2D_3$ (Fig. 8a). The wide spectrum PKC inhibitors GF 109239 X (1 μM) and Gö6076 (1 μM), and the PKC specific inhibitor HBDDE (10 μM) blocked $1\alpha,25(OH)_2D_3$-stimulated activation of ERK-MAP kinase in HL60 cells by ~70% (Fig. 8a). Specific inhibitors of PKCβ (LY379196) and PKCδ (rottlerin) failed to have any effect on the $1\alpha,25(OH)_2D_3$-stimulated activation of ERK-MAP kinase in HL60 cells (Fig. 8a). Buitrago et al. [2003] have shown in smooth muscle cells that $1\alpha,25(OH)_2D_3$-stimulatation of the ERK-MAP kinase cascade involves PKCα-mediated activation of RAF-1 kinase.

Figure 8b shows that the Src tyrosine kinase inhibitor PP1 (1–50 μM) produced a dose-responsive inhibition of $1\alpha,25(OH)_2D_3$-stimulated activation of ERK-MAPK in HL60 cells ($IC_{50} = 11.2 \pm 3.5$ μM). Similarly, the farnesyl transferase inhibitor manumycin A significantly attenuated the $1\alpha,25(OH)_2D_3$-stimulated activation of ERK-MAPK in HL60 cells ($IC_{50} = 21.2 \pm 1.3$ μM, Fig. 8a). RAF kinases are downstream targets of RAS signalling [Kolch, 2000]. RAF (particularly B-RAF) binds to activated RAS which causes a switch in the conformation of the RAF isoform from a closed to an open form. This conformational switch enables RAF to bind to and activate MEK [Kolch, 2000; Terai and Matsuda, 2005]. The

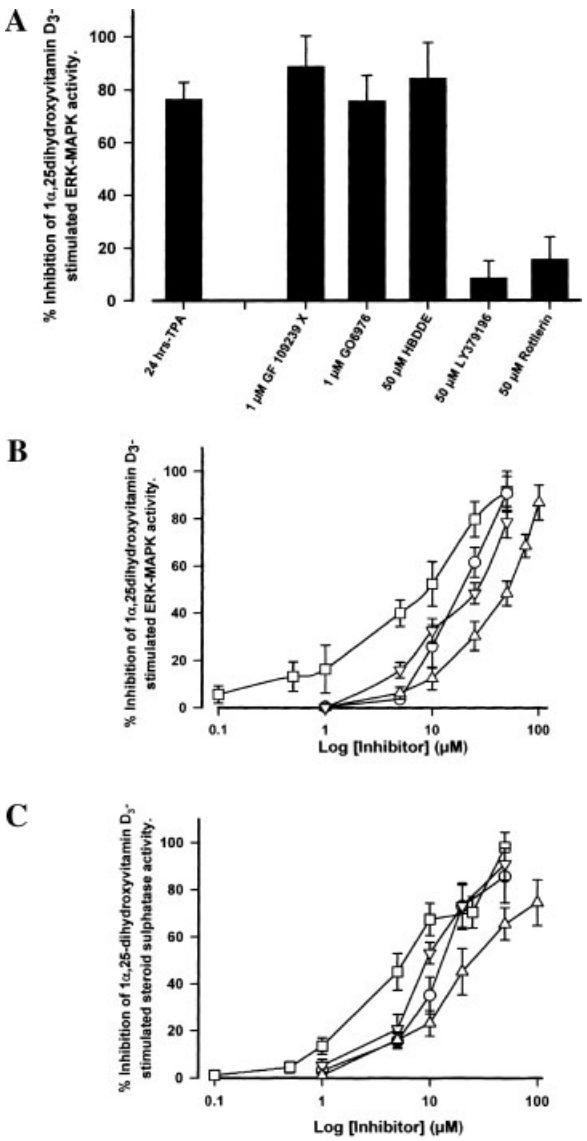

Fig. 8. $1\alpha,25(OH)_2D_3$-stimulated ERK-MAP kinase activity in HL60 cells is dependent on PKCα, Src tyrosine kinase and RAS. **Panel A** shows that the $1\alpha,25(OH)_2D_3$-stimulated ERK-MAP kinase activity in HL60 cells was blocked by inhibition of PKCα, but not PKCβ or PKCδ. HL60 cells were treated TPA (10 nM) for 24 h or the pan-PKC inhibitor GF 109293X (1 μM), the cPKCα/β specific inhibitor Gö 6976 (1 μM), the cPKCα-specific inhibitor HBBDE (50 μM), the cPKCβ inhibitor LY379196 (50 nM) or the cPKCδ,-α-selective inhibitor rottlerrin (50 μM) for 2 h prior to stimulation with 100 nM $1\alpha,25(OH)_2D_3$. After 60 min the cells were harvested/fixed, and total ERK and *p*ERK content measured as described in the Experimental section. Data are shown as the mean ± SEM of the inhibition of $1\alpha,25(OH)_2D_3$-stimulated ERK-MAP kinase activity and were obtained in two experiments performed in triplicate. **Panel B** shows the inhibition of $1\alpha,25(OH)_2D_3$-stimulated ERK-MAP kinase activity in HL60 cells by the Src tyrosine kinase inhibitor PP1 (□), farnesyl transferase inhibitor manumycin A (○) and the RAS-RAF interaction inhibitors sulindac sulphide (△), and RAS inhibitor peptide (▽). Cultures were then treated with inhibitors for 2 h prior to stimulation with 100 nM $1\alpha,25(OH)_2D_3$. After 60 min the cells were harvested/fixed, and total ERK and *p*ERK content measured as described in the Experimental section. Data are shown as the mean ± SEM of the inhibition of $1\alpha,25(OH)_2D_3$-stimulated ERK-MAP kinase activity and were obtained in three experiments performed in quadruplicate. The inhibition curves were fitted to a sigmoidal curve dose response curve and IC_{50}'s were estimated using the 'pharmacology' module of the Sigmaplot™ (version 9.0) graphical software package. **Panel C** shows the inhibition of $1\alpha,25(OH)_2D_3$-stimulated increase in STS activity in HL60 cells by PP1 (□), manumycin A (○), sulindac sulphide (△) and RAS inhibitor peptide (▽). Cultures were treated with the inhibitors for 2 h prior to stimulation with 100 nM $1\alpha,25(OH)_2D_3$. After 24 h the cells were harvested and microsomal STS activity measured as described in the Experimental section. Data are shown as the mean ± SEM of the inhibition of $1\alpha,25(OH)_2D_3$-stimulated STS activity and were obtained in three experiments each performed in quadruplicate. The inhibition curves were fitted to a sigmoidal curve dose response curve and IC_{50}'s were estimated using the 'pharmacology' module of the Sigmaplot™ (version 9.0) graphical software package.

interaction of RAS and RAF can be antagonised by sulindac sulphide [Waldmann et al., 2004] and RAS inhibitor peptide, a mimetic of the RAF–RAS binding domain [Buitrago et al., 2003]. Figure 8b shows that both sulindac sulphide ($IC_{50} = 55.7 \pm 6.9$ μM) and the RAS inhibitory peptide ($IC_{50} = 29.9 \pm 1.9$ μM) produced dose-responsive inhibitions of the 1α,25(OH)$_2$D$_3$-stimulated activation of ERK-MAPK in HL60 cells.

Pharmacological inhibitors were used to investigate whether the RAS-RAF-MEK-MAP-ERK signalling cascade plays a role in the 1α,25(OH)$_2$D$_3$-stimulated increase in STS activity in HL60 and THP-1 cells. Treatment of the myeloid leukaemic cell lines with PP1, manumycin A, or sulindac sulphide or the RAS-inhibitor peptide for longer than 36 h produced unacceptable levels of toxicity. Therefore, the effect of these agents on the 1α,25(OH)$_2$D$_3$ provoked increase in STS activity was measured after 24-h exposure to the seco-steroid. Figure 8c shows that PP1 ($IC_{50} = 6.9 \pm 1.8$ μM), manumycin A ($IC_{50} = 11.9 \pm 1.1$ μM), sulindac sulphide ($IC_{50} = 9.6 \pm 1.2$ μM), and RAS inhibitory peptide ($IC_{50} = 18.4 \pm 2.1$ μM) significantly inhibited the 1α,25(OH)$_2$D$_3$-stimulated increase in STS activity in HL60 cells. Similar results were obtained in THP-1 cells (data not shown).

GW5074 is a selective inhibitor of RAF-1 phosphorylation that inhibits ERK-MAP kinase activation in many cell systems [Chang et al., 2005]. Figure 9a shows that incubating HL60 cells with GW5074 produced a dose-dependent inhibition of 1α,25(OH)$_2$D$_3$-induced ERK-MAP kinase activity ($IC_{50} = 8.9 \pm 2.6$ μM). GW5074 also produced a does responsive inhibition of 1α,25(OH)$_2$D$_3$-stimulated increase in STS activity in HL60 ($IC_{50} = 3.4 \pm 1.3$ μM, Fig. 9a). Similar observations were made using either NB4 ($IC_{50} = 6.7 \pm 2.4$ μM, data not shown) or THP-1 cells ($IC_{50} \sim 35$ μM, data not shown). Phosphorylation of RAF kinase and activation of the ERK-MAP kinase cascade can be inhibited in murine haematopoietic cell lines by chloroquine [Weber et al., 2002]. Figure 9a shows that chloroquine blocked the 1α,25(OH)$_2$D$_3$-stimulated activation of the ERK-MAP kinase pathway in a dose-responsive fashion in HL60 cells ($\sim IC_{50} = 32 \pm 4.6$ μM). The 1α,25(OH)$_2$D$_3$-stimulated increase in STS activity was inhibited by chloroquine in a dose-responsive fashion in HL60 ($IC_{50} = 13.7 \pm 2.7$ μM, Fig. 9b), NB4 ($IC_{50} = 15.7 \pm 3.2$ μM, data not shown) and THP-1 cells ($IC_{50} \sim 50$ μM, data not shown).

ZM 336372 is a specific inhibitor of all three RAF isoforms in cell-free assays. Paradoxically RAF is activated in intact cells by ZM 336372 [Hall-Jackson et al., 1999: Van Gompel et al., 2005]. ZM 336372 (50 μM) produced modest increases in both ERK-MAP kinase activity (Fig. 9b, left hand panel) and STS activity (Fig. 9b, right hand panel) in HL60 cells. Both these effects were abrogated by inhibiting RAF with GW5074 and by the MEK inhibitor PD098059. Figure 9c shows that ZM 336372 produced a dose responsive increase in STS activity in HL60 cells. Furthermore, a synergistic increase in STS was obtained when HL60 cells were co-treated with 100 nM 1α,25(OH)$_2$D$_3$ and increasing concentrations of ZM 336372.

To further investigate the role of the RAS-RAF-MEK cascade in the 1α,25(OH)$_2$D$_3$-stimulated activation of the ERK-MAP kinase signalling pathway in HL60 cells, we treated cells with the KSR-1/MEK inhibitors PD98059 (1–50 μM) and U0126 (0.1–10 μM) [Wang and Studzinski, 2001]. Figure 9a shows that both U0126 ($IC_{50} = 8.6 \pm 1.5$ μM) and PD98059 ($IC_{50} = 45.5 \pm 1.9$ μM) blocked 1α,25(OH)$_2$D$_3$-stimulated activation of the ERK-MAP kinase signalling pathway in a dose-responsive fashion. Conversely, the p38-MAP kinase inhibitor PD169316 and the JNK-MAP kinase inhibitor SP600125 failed to have any effect on the 1α,25(OH)$_2$D$_3$-stimulated activation of ERK-MAP kinase in HL60 cells (Fig. 10a).

Figure 10b shows that the MEK kinase inhibitor PD98059 inhibited the 1α,25(OH)$_2$D$_3$-stimulated increase in STS activity in HL60 cells ($IC_{50} = 6.8 \pm 0.3$ μM), NB4 ($IC_{50} = 7.3 \pm 1.9$ μM) and THP-1 cells ($IC_{50} \sim 100$ μM). Similarly, Figure 9c shows that the structurally unrelated MEK kinase inhibitor U0126 inhibited the 1α,25(OH)$_2$D$_3$-stimulated increase in STS activity in HL60 cells ($IC_{50} = 6.6 \pm 1.3$ μM), NB4 ($IC_{50} = 9.6 \pm 0.2$ μM) and THP-1 cells ($IC_{50} > 100$ μM). The inactive analogue U1024 failed to have any affect on the 1α,25(OH)$_2$D$_3$-stimulated increase in STS activity in any of the myeloid cell lines tested (data not shown).

We treated HL60 cells with an ERK1/2 antisense oligonucleotide (ERK1/2-AS-ODN, 5′-GCCGCCGCCGCCGCAT-3′) for 48 h prior to stimulation with 100 nM 1α,25(OH)$_2$D$_3$. Figure 10d shows that total ERK1/2 protein expression in both HL60 cells was decreased by

>80% after 48 h of treatment with ERK1/2 oligonucleotide antisense when compared with the relevant controls (media alone and the randomly scrambled oligonucleotide R-ODN, 5′-CGCGCGCTCGCGCACCC-3′). The residual ERK1/2 in the ERK1/2-AS-ODN treated cells was still responsive to $1\alpha,25(OH)_2D_3$, and there was no difference in relative levels of activation (control *p*ERK/total ERK = 0.38 ± 0.021, ERK1/2-AS-ODN *p*ERK/total ERK = 0.302 ± 0.026, R-ODN control *p*ERK/total ERK = 0.32 ± 0.048). In the ERK1/2-AS-ODN treated cells, the absolute magnitude of the $1\alpha,25(OH)_2D_3$ activation of ERK1/2 was considerably smaller ($P > 0.03$) than that seen in either the control or R-ODN treated cells. Correspondingly, the $1\alpha,25(OH)_2D_3$-stimulated increase in STS activity was significantly attenuated in the ERK1/2-AS-ODN treated HL60 cells when compared with either the control or R-ODN-treated HL60 cell samples (Fig. 10e).

$1\alpha,25(OH)_2D_3$ stimulates both JNK-MAP [Ji et al., 2002; Wang et al., 2005c] and p38-MAP kinases [Ji et al., 2002] during the monocytic differentiation of myeloid cells. SP600125, acts as a competitive inhibitor at the ATP binding site of JNK-MAP kinase and inhibits $1\alpha,25(OH)_2D_3$-stimulated monocytic differentiation of HL60 cells [Wang et al., 2005c]. SP600125 produced a dose-responsive inhibition of the $1\alpha,25(OH)_2D_3$-stimulated increase in STS activity in HL60, NB4 and THP-1 cells (IC_{50}'s = 8.7 ± 1.9 μM, 22.4 ± 5.1 μM and 27.2 ± 3.2 μM

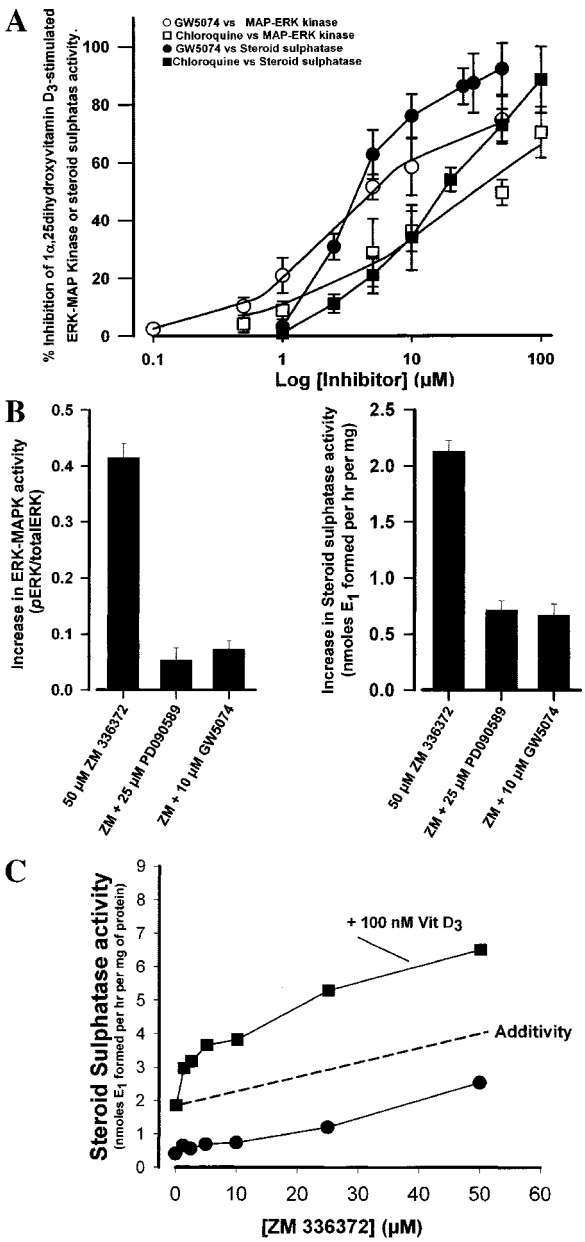

Fig. 9. RAF kinase is involved in the $1\alpha,25(OH)_2D_3$-stimulated increase in MAP-ERK kinase and STS activity in HL60 cells. **Panel A** shows that the RAF kinase inhibitors GW5074 (○,●) and chloroquine (□,■) block both the $1\alpha,25(OH)_2D_3$-stimulated increase in ERK-MAP kinase activity (open figures) and STS activity (closed figures) in HL60 cells. Cultures were treated with either GW5074 or chloroquine for 2 h prior to stimulation with 100 nM $1\alpha,25(OH)_2D_3$. For the measurement of ERK-MAP kinase activity, the cells were harvested/fixed after 60 min stimulation and total ERK and *p*ERK content measured as described in the Experimental section. Data are shown as the mean ± SEM of the inhibition of $1\alpha,25(OH)_2D_3$-stimulated ERK-MAP kinase activity and were obtained in three experiments performed in quadruplicate. For measurement of STS activity, cultures were stimulated with 100 nM $1\alpha,25(OH)_2D_3$ for 24 h. Microsomal STS activity measured as described in the Experimental section. Data are shown as the mean ± SEM of the inhibition of $1\alpha,25(OH)_2D_3$-stimulated STS activity and were obtained in three experiments performed in quadruplicate. The inhibition curves were fitted to a sigmoidal curve dose response curve and IC_{50}'s estimated using the 'pharmacology' module of the Sigmaplot™ (version 9.0) graphical software package. **Panel B** shows that the 'RAF-activator' ZM 336377 (50 μM) stimulates both ERK-MAP kinase activity (left hand panel) and STS activity (right hand panel) in HL60 cells. Panel B also shows that the ZM 336377 stimulated increases in ERK-MAP kinase activity and STS activity were blocked by pre-treating the cells with the MEK inhibitor PD90589 (25 μM) and the RAF inhibitor GW5074 (10 μM). Changes in ERK-MAP kinase activity and STS activity were measured as described above. Data were obtained in three experiments each performed in triplicate. **Panel C** shows that ZM 336372 (●, 1–50 μM) produced a dose responsive increase in STS activity in HL60 cells. Furthermore, panel C shows that co-treatment of HL60 cells with 100 nM $1\alpha,25(OH)_2D_3$ and increasing concentrations of ZM 336372 (■) produces a synergistic increase in STS activity. Cells were treated with $1\alpha,25(OH)_2D_3$ and/or ZM 336372 for 72 h. Microsomal STS activity measured as described in the Experimental section. Data were obtained in six separate experiments each performed in triplicate.

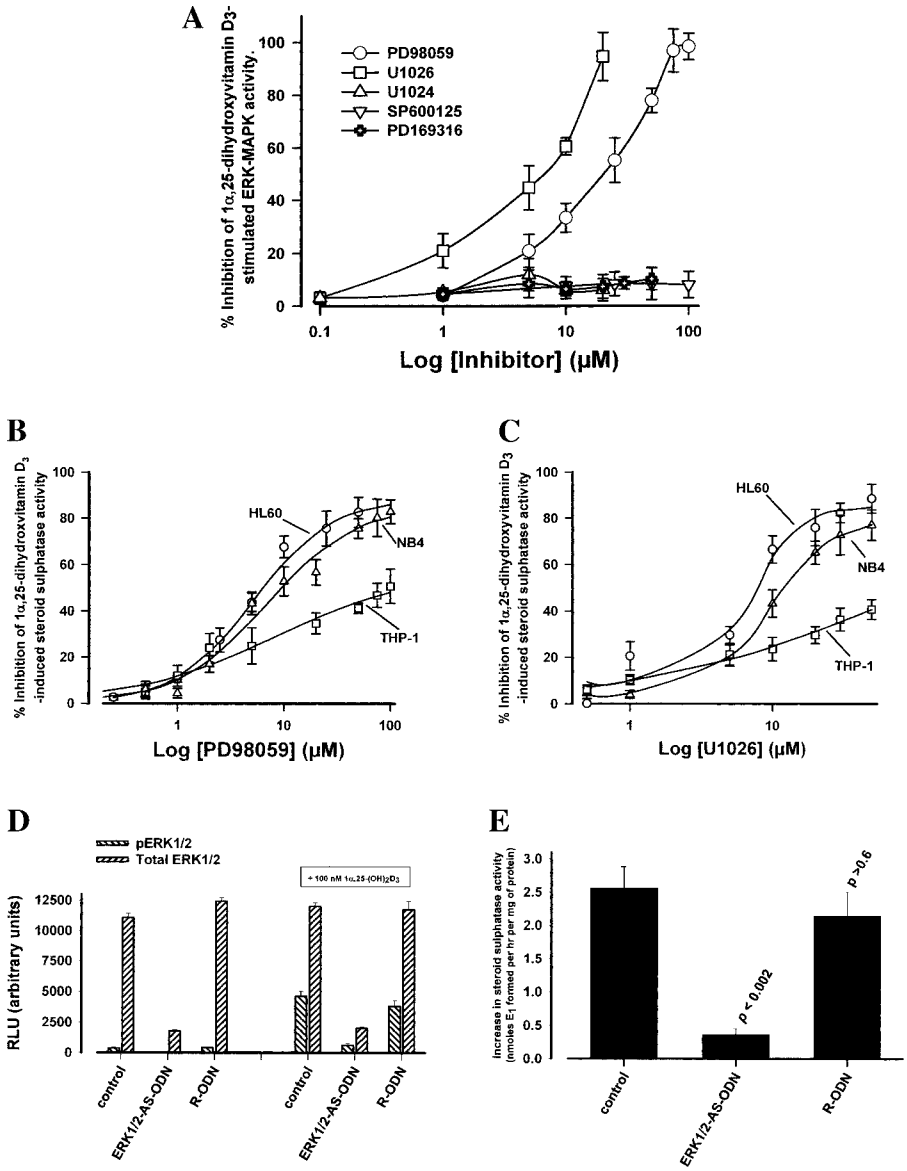

Fig. 10. Inhibition of ERK-MAP kinase blocks the 1α,25(OH)$_2$D$_3$-stimulated increase in STS activity in HL60 cells. **Panel A** shows that the MEK inhibitors PD98059 (○) and U1026 (□) inhibit 1α,25(OH)$_2$D$_3$-stimulated ERK-MAP kinase activity in HL60 cells. Cells were treated with increasing concentrations of either PD98059 and or U1026 for 2 h prior to stimulation with 100 nM 1α,25(OH)$_2$D$_3$. After 60 min reactions were quenched and ERK-MAP kinase activity measured as described in the Methods section. 1α,25(OH)$_2$D$_3$-stimulated ERK-MAP kinase activity was not inhibited by the JNK-MAP kinase inhibitor SP600125 (▽) or the p38-MAP kinase inhibitor PD169316 (◇). Data are shown as the mean ± SEM of the inhibition of 1α,25(OH)$_2$D$_3$-stimulated ERK-MAP kinase activity and were obtained in three experiments each performed in triplicate. PD98059 (**panel B**) and U1026 (**panel C**) inhibit the 1α,25(OH)$_2$D$_3$-stimulated increase in STS activity in HL60 (○), NB4 (△) and THP-1 (□) cells. Cells were treated with increasing concentrations of either PD98059 and or U1026 for 2 h prior to stimulation with 100 nM 1α,25(OH)$_2$D$_3$. After 72 h the cells were harvested and microsomal STS activity was measured as described in the Methods section. Data are shown as the mean ± SEM of the inhibition of the 1α,25(OH)$_2$D$_3$-stimulated increase in STS activity and were obtained in three experiments each performed in triplicate. The inhibition curves were fitted to a sigmoidal curve dose-response curve and IC$_{50}$'s estimated using the 'pharmacology' module of the Sigmaplot™ (version 9.0) graphical software package. Treatment of HL60 cells with an ERK1/2 antisense oligonucleotide downregulates the expression of ERK-MAP kinase (**panel D**) and blocks the 1α,25(OH)$_2$D$_3$-stimulated increase in STS activity (**panel E**). Cell cultures were treated for 24 h with a phosphorothioate-modified ERK1/2-MAP kinase antisense oligodeoxynucleotide (5 μM, ERK1/2-AS-ODN) or with a randomly scrambled oligodeoxynucleotide (5 μM, R-ODN). After treatment cell cultures were washed twice in pre-warmed tissue culture medium, resuspended and stimulated with 100 nM 1α,25(OH)$_2$D$_3$ for either 60 min (ERK-MAP kinase assay) or for 72 h (STS assay). ERK-MAP kinase and STS activity were measured as described in the Experimental section. Data were obtained in two experiments each performed in triplicate.

respectively, data not shown). In contrast, the p38-MAPK inhibitor PD169316 did not have a major effect on the $1\alpha,25(OH)_2D_3$-stimulated increase in STS activity in HL60, NB4 or THP-1 cells. The maximum inhibition produced by 50 μM PD169316 was <30% (data not shown).

DISCUSSION

We have previously shown that an increase in STS activity is a marker of $1\alpha,25(OH)_2D_3$-stimulated monocytic differentiation in HL60 cells [Hughes et al., 2001, 2005]. Here, we show that a VDR_{nuc}-mediated activation of the ERK-MAP kinase pathway plays an important role in the $1\alpha,25(OH)_2D_3$-stimulated increase in STS activity in HL60 cells, and the related U937 and THP-1 myeloid leukaemic cell lines.

$1\alpha,25(OH)_2D_3$ failed to stimulate an increase in STS activity in the KG-1a and KG-1 cell lines (Fig. 1b), primary FAB-M0 blast cells (data not shown) and the K562 primitive erythro-leukaemic cell line (data not shown). $1\alpha,25(OH)_2D_3$ also failed to increase PLD activity in KG-1 and KG-1a cell lines (Fig. 3b). El Marjou et al. [2000] have previously demonstrated that PLD protein expression was stimulated by $1\alpha,25(OH)_2D_3$ in mature leukaemic cell lines but not in primitive leukaemic blast cells. At a whole cell level, KG-1a and KG-1 cells fail to growth arrest or acquire other markers of monocytic differentiation (CD11b or CD14 surface expression, phagocytosis of zymosan coated yeast or the ability to reduce NBT) even when treated with supra-physiological concentrations of $1\alpha,25(OH)_2D_3$ [Hughes and Brown, unpublished data].

Why do immature myeloid leukaemic cell lines fail to upregulate the expression of differentiation-related markers in response to $1\alpha,25(OH)_2D_3$? The lack of responsiveness of KG-1a and KG-1 cells is not related to a lack of VDR_{nuc} expression since low levels of the receptor are found in these cell types [Manfredini et al., 1999]. Furthermore, in both cell types the liganded VDR_{nuc} can associate with RXR and drive the transactivation of transfected VDRE-containing reporter constructs and endogenous 'house keeping genes' but not 'differentiation-related' genes [Manfredini et al., 1999]. For expression of 'differentiation-related' genes in myeloid cells transcription factor/co-regulator complexes have to access critical regulatory elements on DNA [Rosmarin et al., 2005]. This is dependent on the chromatin microenvironment and chromatin structure appears to change during monocytic/macrophage differentiation. A chromatin structure that 'allows' transcription of 'differentiation related genes' only develops during the latter stages of the lineage specification process [Tagoh et al., 2004]. The promoter regions of the *STS* and *PLD* genes, as well as other 'differentiation-related' genes appear to be inaccessible to the transcription machinery in KG-1 and KG-1a cells.

Co-activation of non-genomic signalling pathways (e.g. PKCα, PLD RAF, ERK-MAP kinase) seems to be essential for $1\alpha,25(OH)_2D_3$-stimulated monocytic differentiation. These pathways appear to be either defective or absent in primitive haematopoietic cells. For example, a rapid increase in the activities of both PLD and ERK-MAP kinase is not seen in $1\alpha,25(OH)_2D_3$-stimulated KG-1a and KG-1 cells, but is observed in the more mature myeloid leukaemic cell lines. Others have shown that TNF-α activates the ERK-MAP kinase signalling cascade in mature myeloid leukaemic cells [Andersson and Sundler, 2000], but not in KG-1a and KG-1 cells [Hu et al., 1999]. Several PKC isoforms, including PKCα, are expressed at very low levels in KG-1a and KG-1 cells [Hooper et al., 1989; MacFarlane and Manzel, 1994], and this may contribute to a failure of these cells to differentiate in response to $1\alpha,25(OH)_2D_3$. Refractoriness of KG-1a and KG-1 cells to differentiation-inducing stimuli appears to lie in defects in the initiation, propagation, and integration of both the genomic and non-genomic signalling cues that drive differentiation.

An increase in the expression/activity of PLD appears to play an important role in monocytic and granulocytic differentiation of myeloid leukaemic cell lines [Collado-Escobar and Mollinedo, 1994; Ohguchi et al., 1997; Nakashima et al., 1998; Burke et al., 1999; El Marjou et al., 2000; Hsu et al., 2000; Neri et al., 2002; Kang et al., 2004; Di Fulvio and Gomez-Cambronero, 2005]. $1\alpha,25(OH)_2D_3$ has a complex effect on PLD activity in HL60 cells. There is an initial transient and rapid non-genomic stimulation of PLD activity. This is followed by a slower and more persistent increase in the expression of PLD. Others have described a late increase in PLD expression during monocytic differentiation of myeloid leukaemic cell lines [El Marjou et al., 2000]. Our study shows that PLD is involved in $1\alpha,25(OH)_2D_3$-mediated stimulation of STS

activity in HL60 cells: (i) there is a positive correlation between basal activities of both PLD and STS, (ii) in lines that 1α,25(OH)$_2$D$_3$ failed to stimulate an increase in STS activity, the secosteroid also failed to stimulate PLD and (iii) pharmacological inhibition of PLD activity blocks the 1α,25(OH)$_2$D$_3$-mediated increases in STS activity.

Augmentation of PLD activity increases the availability of the second messenger PA [Exton, 2002]. PA had no significant effect on either basal or 1α,25(OH)$_2$D$_3$-stimulated STS activity in HL60 cells. PA can be formed by the phosphorylation of DAG by DGK [Kanoh et al., 2002]. R59022, a pan-DGK inhibitor, failed to have any affect on the 1α,25(OH)$_2$D$_3$-stimulated increase in STS activity in HL60, U937 and THP-1 cells. These observations suggest that PA does not play a major role in 1α,25(OH)$_2$D$_3$ signalling in myeloid cells. In fact, DGKγ is downregulated during monocytic differentiation in HL60 and U937 cells, and overexpression of constitutively-active DGKγ blocks monocytic differentiation [Yamada et al., 2003]. These observations suggest that a PA metabolite plays an important role in driving monocytic differentiation. Phosphatidic acid phosphohydrolase (PAPH) converts PA into DAG. PAPH activity increases during the DMSO-induced differentiation of U937 promyelocytic cells, and the PLD-PAPH pathway is the major source of DAG in these cells [Hsu et al., 2000]. The 1α,25(OH)$_2$D$_3$-stimulated increase in STS activity in HL60 cells is blocked by PAPH inhibitors (propranolol and chlorpromazine). Therefore, generation of DAG via the PLD-PAPH pathway plays an important role in the 1α,25(OH)$_2$D$_3$-stimulated increase in STS activity in HL60 cells.

The most studied DAG targets are the calcium dependent, DAG-dependent conventional protein kinase C (PKC) isoforms (α, βI, βII and γ) and the calcium-independent, DAG-dependent novel PKC (nPKC) isoforms (δ, ε, η, θ) [Yang and Kazanietz, 2003]. HL60 cells express most of the known PKC isoforms [Miguel et al., 2000]. Translocation of DAG-sensitive PKC isoforms to the plasma membrane or the nucleus is essential for 1α,25 (OH)$_2$D$_3$-mediated monocytic differentiation of myeloid leukaemic cell lines [MacFarlane and Manzel, 1994; Berry et al., 1996; Pan et al., 1997; Slosberg et al., 2000]. The pan PKC activator TPA produced a transient increase in STS activity in HL60 cells. Thymelaeatoxin, a specific activator of PKCα had a small stimulatory effect on STS activity and potentiated the 1α,25(OH)$_2$D$_3$-stimulated increase in STS activity in HL60 cells. Activation of PKCβ or PKCδ, with bistratene A and DOPPA respectively, had no effect on basal or 1α,25(OH)$_2$D$_3$-stimulated STS activity. Downregulation of PKC, by prolonged incubation with TPA, and wide specificity inhibitors blocked 1α,25(OH)$_2$D$_3$-stimulated STS activity. Pharmacological inhibitors of PKCα, but not of PKCβ or PKCδ, abrogate the 1α,25(OH)$_2$D$_3$-stimulated increase in STS activity in HL60 cells.

PKCα is one of the major inputs required for activation of the RAF kinase family [Kolch, 2000]. Members of the RAF family are MAP kinase kinase kinases (MAP3K), which phosphorylate the MAP kinase kinase MEK which, in turn, activates the MAP kinase ERK1/2 [Kolch, 2000; Pearson et al., 2000]. PA facilitates the association of RAF with the plasma membrane associations and increases RAF kinase activity. Studzinski et al. [2005] have recently shown that RAF-1 is phosphorylated when HL60 cells are stimulated with 1α,25(OH)$_2$D$_3$. Furthermore, phosphorylation of RAF-1 is essential to both the activation of the ERK-MAP kinase cascade and for the successful monocytic differentiation of HL60 cells [Studzinski et al., 2005]. We have not directly assessed PKCα-mediated phosphorylation of RAF, but Figure 8a shows that inhibition of protein kinase Cα, but not the PKCβ or PKCδ, abrogates the 1α,25(OH)$_2$D$_3$-stimulated increase in ERK-MAPK activity. This corroborates earlier findings that activation of PKCα by 1α,25(OH)$_2$D$_3$ is essential for stimulation of ERK-MAP kinase activity in HL60 cells [Marcinkowska et al., 1997] and rat smooth muscle cells [Buitrago et al., 2003]. Association with RAS at the plasma membrane is also necessary for the activation of RAF [Kolch, 2000]. To this end, the 1α,25(OH)$_2$D$_3$-stimulated increase in ERK-MAPK activity was inhibited by agents which interfere with the membrane localisation of RAS (manumycin A) or that physically block the interaction of RAS with RAF (sulindac sulphide and RAS inhibitory peptide). That RAF inhibitors (GW5074, choloroquine) block and the RAF activator ZM336372 augment, the 1α,25(OH)$_2$D$_3$-stimulated increase in ERK-MAP kinase and STS activity in HL60 cells highlights the importance

of RAF. Similarly, pharmacological or antisense inhibition of ERK-MAP kinase activity blocks the $1\alpha,25(OH)_2D_3$-stimulated increase in ERK-MAP kinase and STS activity in HL60 cells.

The data demonstrating that VDR_{nuc}, but not RXR, antagonists blocks the $1\alpha,25(OH)_2D_3$-stimulated increase in ERK-MAP kinase activity implies a direct role for VDR_{nuc} monomer in activating ERK-MAP kinase, thereby adding to the growing list of non-genomic signalling pathways that are switched on by VDR_{nuc}. For example the VDR_{nuc} has been shown to associate with and/or activate RAF [Studzinski et al., 2005], the serine/threonine protein phosphatases PP1c and PP2Ac [Bettoun et al., 2004], p70 S6 kinase [Bettoun et al., 2004], and phosphatidylinositol 3-kinase [Hmama et al., 1999] in myeloid leukaemic cell lines and the tyrosine kinase Src [Buitrago et al., 2003; Capiati et al., 2004; Rossi et al., 2004; Vertino et al., 2005], and guanylate cyclase [Barsony and McKoy, 1992] in other cell types. A model has been developed in the Norman laboratory, which suggests that when liganded the VDR_{nuc} can adopt multiple conformations depending on the nature of the ligand. When the VDR_{nuc} is liganded by $1\alpha,25(OH)_2D_3$ in its 6-*s*-*cis* configuration the receptor adopts a conformation allowing it to couple to molecules regulating the activation of non-genomic signalling pathways [Norman et al., 2004]. It is likely that when the VDR_{nuc} is liganded by ZK159222 or ZK168281, it adopts an alternate conformation that does not allow it to couple to the intracellular signalling pathways. Free unliganded VDR_{nuc} is largely cytoplasmic but when liganded rapidly redistributes about the cell, predominately to the nucleus [Racz and Barsony, 1999]. However, significant quantities of liganded-VDR_{nuc} have been found associated with either the endoplasmic reticulum or the plasma membrane in a osteoclast cell line [Kim et al., 1996]. In more recent studies, the translocation of a small fraction of the cellular VDR_{nuc} from the nucleus to the plasma membrane has been reported in both avian skeletal muscle cells [Capiati et al., 2002] and the MCF-7 breast cancer cell line following $1\alpha,25(OH)_2D_3$ stimulation [Capiati et al., 2004]. An intact microtubular system is required for the nuclear-plasma membrane translocation of the VDR_{nuc} [Capiati et al., 2002, 2004]. More specifically VDR_{nuc} has been reported to localise to a cholesterol rich membrane fraction (CMF) in the plasma membrane of chick, rat and mouse intestinal cells, mouse lung and kidney cells, a rat osteoblast-like cell line [Norman et al., 2002; Huhtakangas et al., 2004; Bula et al., 2005] and, of particular relevance to this study, the NB4 human myeloid leukaemic cell line [Huhtakangas et al., 2004]. Cholesterol-rich microdomains are highly specialised areas of the plasma membranes since the adaptor, scaffolding or effector molecules required for coupling receptors to intracellular signalling pathways are localised in CMF. Therefore, in theory the VDR_{nuc} could be located close to the machinery it requires to activate non-genomic signalling pathways. Studies are underway to confirm this possibility.

We have previously shown that $1\alpha,25(OH)_2D_3$ increases expression of the mRNA encoding STS in HL60 cells [Hughes et al., 2001]. The $1\alpha,25(OH)_2D_3$-stimulated increase in STS activity is blocked by the RXR antagonist VTP195393 [Hughes et al., 2005], suggesting the involvement of a VDR_{nuc}/RXR component. However, this does not necessarily mean that the *STS* gene is a direct $1\alpha,25(OH)_2D_3$ target. Microarray experiments have shown that $1\alpha,25(OH)_2D_3$ stimulates the expression of >900 genes in SCC25 human oral squamous carcinoma cells. Only 65% of these genes have a functional VDRE in their promoter [Wang et al., 2005]. The promoter region of the *STS* gene has been identified [Li et al., 1996] and it does not contain a canonical VDRE [data not shown].

Stimulation of STS activity/expression by $1\alpha,25(OH)_2D_3$ in HL60 and other myeloid cells may well be independent of VDRE-mediated events and may well be driven $1\alpha,25(OH)_2D_3$/VDR_{nuc}-mediated activation of non-genomic signalling pathways such as the ERK-MAP kinase cascade. It is well established that MAP kinase pathways play important roles in regulating eukaryotic gene expression in response to extracellular stimuli by acting as (i) structural adaptors allowing the proper formation of transcription factor/activator complexes and (ii) enzymatic activators of the transcription machinery [Edmunds and Mahadevan, 2004]. In particular, it has been shown that ERK-MAP kinase stimulates both the DNA binding ability and the transcriptional activity of the NF-κB and C/EBPβ transcription factors. $1\alpha,25(OH)_2D_3$ stimulates an increase in the DNA binding and transcriptional activity of both C/EBPβ [Christakos et al., 2003; Ji and Studzinski, 2004; Studzinski et al., 2005] and NF-κB [Berry

et al., 2002; Kim et al., 2002] in myeloid cells. C/EBPβ DNA binding capacity and transcriptional activity is increased by phosphorylation by ERK-MAP kinase or p90 Rsk kinase in myeloid and other cell types [Cieslik et al., 2005; Studzinski et al., 2005; Tang et al., 2005; Wang et al., 2005]. In fact, activation of the ERK-MAP kinase-p90 Rsk kinase-C/EBPβ signalling cassette is essential to the 1α,25(OH)$_2$D$_3$-stimulated increase in the expression of CD14 in HL60 cells [Studzinski et al., 2005]. One possible mechanism for the activation of the p65 subunit of NF-κB is phosphorylation by members of ERK-MAP kinase/MSK1/p90 Rsk kinase signalling complex [Kim et al., 2001; Vermeulen et al., 2002]. Computer analysis has identified several potential NF-κB and C/EBPβ binding sites in the STS promoter [data not shown]. Stimulation of the ERK-MAP kinase activated pathways by 1α,25(OH)$_2$D$_3$ could contribute to the increase in steroid sulphates activity by activating C/EBPβ and/or NF-κB.

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REFERENCES

Andersson K, Sundler R. 2000. Signalling to translational activation of tumour necrosis factor-α expression in human THP-1 cells. Cytokine 12:1784–1787.

Bae Y, Lee T, Park J, Hut J, Kim Y, Heo K, Kwak J. 2003. Identification of a compound that directly stimulates phospholipase C activity. Mol Pharmacol 63:1043–1050.

Barletta F, Dhawan P, Christakos S. 2004. Integration of hormone signalling in the regulation of human 25(OH)D$_3$ 24 hydroxylase transcription. Am J Physiol Endocrinol Metab 286:E595–E608.

Batista E, Warbington M, Badwey J, Van Dyke T. 2005. Differentiation of HL-60 cells to granulocytes involves regulation of select diacylglycerol kinases (DGKs). J Cell Biochem 94:774–793.

Barsony J, McKoy W. 1992. Molybdate increases intracellular 3′,5′-guanosine cyclic monophosphate and stbilizes vitamin D receptor association with tubulin-containing filaments. J Biol Chem 267:24457–24465.

Berry D, Antochi R, Bhatia M, Meckling-Gill K. 1996. 1,25-dihydroxyvitamin D$_3$ stimulates expression and translocation of protein kinase Cα and Cδ via a non-genomic mechanism and rapidly induces phosphorylation of a 33-kDA protein in acute promyelocytic NB4 cells. J Biol Chem 271:16090–16096.

Berry D, Clark C, Meckling-Gill K. 2002. 1α,25-dihydroxyvitamin D$_3$ stimulates phosphorylation of IκBα and synergises with TPA to induce nuclear translocation of NFκB during monocytic differentiation of NB4 leukemic cells. Exp Cell Res 272:176–184.

Bertagnolo V, Neri L, Marchisio M, Mischiati C, Capitani S. 1999. Phosphoinositide 3-kinase activity is essential for all-*trans*-retinoic acid-induced granulocytic differentiation of HL-60 cells. Canc Res 59:542–546.

Bertagnolo V, Brugnoli F, Marchisio M, Celeghini C, Carini C, Capitani S. 2004. Association of PI 3-K with tyrosine phosphorylated VAV is essential for its activity in neutrophil-like maturation of myeloid cells. Cell Signal 16:423–433.

Bettoun D, Lu J, Khalifa B, Yee Y, Chin W, Nagpal S. 2004. Ligand modulates VDR-Ser/Thr protein phosphatase interaction and p70S6 kinase phosphorylation in a cell-context-dependent manner. J Steroid Biochem Mol Biol 89:195–198.

Bhatia M, Kirkland J, Meckling-Gill K. 1995. Monocytic differentiation of acute promyelocytic leukaemia cells in response to 1,25-dihydroxyvitamin D$_3$ is independent of nuclear receptor binding. J Biol Chem 270:15692–15965.

Boland R, Buitrago C, De Boland A. 2005. Modulation of tyrosine phosphorylation signalling pathways by 1α,25(OH)$_2$-vitamin D$_3$. Trend Endocrinol Metab 16: 280–287.

Buitrago C, Pardo V, De Boland A, Boland R. 2003. Activation of RAF-1 through Ras and protein kinase Cα mediates 1α,25(OH)$_2$-vitamin D$_3$ regulation of the mitogen-activated protein kinase pathway in muscle cells. J Biol Chem 278:2199–2205.

Bula C, Huhtakangas J, Olivera C, Bishop J, Norman A, Henry H. 2005. Presence of a truncated form of the VDR in a strain of VDR-KO mice. Endocrinology 146:5581–5586.

Bulayeva N, Gametchu B, Watson C. 2004. Quantitative measurement of estrogen-induced ERK 1 and 2 activation via multiple membrane-initiated signalling pathways. Steroids 69:181–192.

Burke J, Davern L, Gregor K, Owczarczak L. 1999. Differentiation of U937 cells enables a phospholipase D-dependent pathway of cytosolic phospholipase A2 activation. Biochem Biophys Res Commun 260:232–239.

Cambien B, Millet M-A, Schmid-Antomarchi H, Brosette N, Rossi B, Schmid-Alliana A. 1999. Src-regulated extracellular-regulated kinase and syk-regulated c-jun N-terminal kinase pathways act in conjunction to induce IL-1 synthesis in response to microtubule disruption in HL60 cells. J Immunol 163:5079–5085.

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.

Capiati D, Rossi A, Picotti G, Benassati S, Boland R. 2004. Inhibition of serum-stimulated mitogen activated protein kinase by 1α,25(OH)$_2$-vitamin D$_3$ in MCF-7 breast cancer cells. J Cell Biochem 93:384–397.

Carlberg C. 2003. Molecular basis of the selective activity of vitamin D analogues. J Cell Biochem 88:274–281.

Chang M, Chen B, Yu M, Sheu J, Chen T, Lin C. 2005. Phorbol 12-myristate 13-acetate upregulates cyclooxygenase-2 expression in human pulmonary epithelial cells via Ras, Raf-1, ERK and NF-κB, but not p38 MAPK, pathways. Cell Signal 17:299–310.

Chen W, Thiboutot D, Zoublis C. 2002. Cutaneous androgen metabolism: Basic research and clinical perspectives. J Invest Dermatol 119:992–1007.

Christakos S, Barletta F, Huenning M, Dhawan P, Liu Y, Porta A, Peng X. 2003. Vitamin D target proteins: Function and regulation. J Cell Biochem 88:238–244.

Cieslik K, Zhu Y, Shtivelband M, Wu K. 2005. Inhibition of p90 ribosomal S6 kinase-mediated CCAAT/enhancer-binding protein-β activation and cyclooxygenase expression by salicylate. J Biol Chem 280:18411–18417.

Collado-Escobar D, Mollinedo F. 1994. Dexamethasone modifies the functional responses of the granulocytic differentiating HL-60 cells. Biochem J 299:553–559.

Compagnone N, Salido E, Shapiro L, Mellon S. 1997. Expression of steroid sulfatase during embryogenesis. Endocrinology 138:4768–4773.

Davies S, Reddy H, Caivano M, Cohen P. 2000. Specificity and mechanism of action of some commonly used protein kinase inhibitors. Biochem J 351:95–105.

Di Fulvio M, Gomez-Cambronero J. 2005. Phospholipase D (PLD) gene expression in human neutrophils and HL-60 differentiation. J Leukoc Biol 77:999–1007.

Dominguez O, Valencia S, Loza A. 1975. On the role of steroid sulfates in hormone biosynthesis. J Ster Biochem 6:301–309.

Dusso A, Brown A, Slatopolsky E. 2005. Vitamin D. Am J Physiol Renal Physiol 289:F8–F28.

Dwivedi P, Hii C, Ferrante A, Tan J, Der C, Omgahl J, Morris H, May B. 2002. Role of MAP kinases in the 1,25-dihydroxyvitamin D_3-mediated transactivation of the rat cytochrome p450C24 (CYP24) promoter. Specific functions for ERK1/ERK2 and ERK5. J Biol Chem 27:29643–29653.

Ebisuya M, Kondoh K, Nishida E. 2005. The duration, magnitude and compartmentalization of ERK MAP kinase activity: Mechanisms for providing signalling specificity. J Cell Science 118:2997–3002.

Edmunds J, Mahadevan L. 2004. MAP kinases as structural adaptors and enzymatic activators in transcription complexes. J Cell Sci 117:3715–3723.

Edwards D. 2005. Regulation of signal transduction pathways by estrogen and progesterone. Annu Rev Physiol 67:335–376.

El Marjou M, Montalescot V, Buzyn A, Geny B. 2000. Modifications in phospholipase D activity and isoform expression occur upon maturation and differentiation in vivo and in vitro in human myeloid cells. Leukemia 14: 2118–2127.

Exton J. 2002. Regulation of phospholipase D. FEBS Lett 531:58–61.

Faccinetti M, Boland R, De Boland A. 1998. Calcitrol transmembrane signalling: Regulation of rat muscle phospholipase D activity. J Lipid Res 39:197–204.

Farach-Carson M, Davis P. 2003. Steroid hormone interactions with target cells: Cross talk between membrane and nuclear pathways. J Pharmacol Exp Ther 307:839–845.

Feuerstein N, Cooper H. 1984. Rapid phosphorylation-dephosphorylation of specific proteins induced by phorbol ester in HL-60 cells. Further characterization of the phosphorylation of 17 kilodalton and 27-kilodalton proteins in myeloid leukaemic cells and human monocytes. J Biol Chem 259:2782–2788.

Fleet J. 2004. Rapid, membrane-initiated actions of 1,25 dihydroxyvitamin D: What are they and what do they mean? J Nutr 134:3215–3218.

Fong W-F, Tse A, Poon K-H, Wang C. 2005. Magnolol and honokiol enhance HL-60 human leukaemia cell differentiation induced by 1,25-dihydroxyvitamin D_3 and retinoic acid. Internal. J Biochem Cell Biol 37:427–441.

Gardner J, Balsubramanyam M, Studzinski G. 1997. Up-regulation of Ca^{2+} influx mediated by store-operated channels in HL60 cells induced to differentiate by 1α,25-dihydroxyvitamin D_3. J Cell Physiol 172:284–295.

Glasow A, Prodromou N, Xu K, Von Lindern M, Zelent A. 2005. Retinoids and myelomonocytic growth factors cooperatively activate RARα and induce human myeloid leukaemia cell differentiation via MAP kinase pathways. Blood 105:341–349.

Grande A, Montanari M, Tagliafico E, Manfredini R, Marani T, Siena M, Tenedini E, Gallinelli A, Ferrari S. 2002. Physiological levels of 1α,25-dihydroxyvitamin D_3 induce the monocytic commitment of $CD34^+$ hematopoietic progenitors. J Leukoc Biol 71:641–651.

Griffiths G, Garrone B, Deacon E, Owen P, Pongracz J, Mead G, Bradwell A, Watters D, Lord J. 1996. The polyether Bistratene A activated protein kinase C-δ and induces growth arrest in HL60 cells. Biochem Biophys Res Commun 22:802–808.

Grynkiewicz G, Poenie M, Tsien R. 1985. A new generation of Ca^{2+}indicators with greatly improved fluorescence properties. J Biol Chem 260:3440–3450.

Hall-Jackson C, Eyers P, Cohen P, Goedert M, Boyle F, Hewitt N, Plant H, Hedge P. 1999. paradoxical activation of Raf by a novel Raf inhibitor. Chem Biol 6:559–568.

Hanley K, Jiang Y, Katagiri C, Feingold K, Williams M. 1997. Epidermal steroid sulfatase and cholesterol sulfotransferase are regulated during late gestation in the fetal rat. J Invest Dermatol 108:871–875.

Hanson S, Best M, Wong C-H. 2004. Sulphatases: Structure, mechanism, biological activity, inhibition and synthetic utility. Angew Chem Int Ed 43:5736–5763.

Hara H, Yasunami Y, Adachi T. 2002. Alteration of cellular phosophorylation state affects vitamin D receptor-mediated CYP3A4 mRNA induction in Caco-2 cells. Biochem Biophys Commun 296:182–188.

Hmama Z, Nandan D, Sly L, Knutson K, Herrera-Velit P, Reiner N. 1999. 1α,25-dihydroxyvitamin D_3-induced myeloid cell differentiation is regulated by a vitamin D receptor-phosphatidylinositol 3-kinase signalling complex. J Exp Med 190:1583–1594.

Holick M. 2003. Vitamin D: A millennium perspective. J Cell Biochem 88:296–307.

Hooper W, Abraham R, Ashendel C, Woloschak G. 1989. Differential responsiveness to phorbol esters correlates with differential expression of protein kinase Cin KG-1 and KG-1a human myeloid leukaemia cells. Biochim Biophys Acta 1013:47–54.

Hsu F, MA Z, Wohltmann M, Bohrer A, Nowatzke W, Rananadham S, Turk J. 2000. Electrospray ionization/mas spectrometric analyses of human promonocytic

U937 cell glycerolipids and evidence that differentiation is associated with membrane lipid composition changes that facilitate phospholipase A2 activation. J Biol Chem 275:16579–16589.

Hu T, Exton J. 2005. 1-Butanol interferes with phospholipase D1 and protein kinase Cα association and inhibits phospholipase D1 basal activity. Biochem Biophys Res Commun 327:1047–1051.

Hu X, Tang M, Fisher A, Olashaw N, Zuckerman K. 1999. TNF-α-induced growth suppression of CD34$^+$ myeloid leukemic cell line signals through TNF receptor type I and is associated with NF-κB activation. J Immunol 163: 3106–3115.

Hughes P, Twist L, Durham J, Choudhry M, Drayson M, Chandraratna R, Michell R, Kirk C, Brown G. 2001. Up-regulation of steroid sulphatase activity in HL60 promyelocytic cells by retinoids and 1α25-dihydroxyvitamin D$_3$. Biochem J 365:361–371.

Hughes P, Steinmeyer A, Chandraratna R, Brown G. 2005. 1α,25-dihydroxyvitamin D$_3$ stimulates steroid sulphatase activity in HL60 and NB4 acute myeloid leukaemia cell lines by different receptor-mediated mechanisms. 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.

Humeniuk-Polaczek R, Marinkowska E. 2004. Impaired nuclear localization of vitamin D receptor in leukemia cells resistant to calcitriol-induced differentiation. J Steroid Biochem Mol Biol 88:361–366.

Ji Y, Studzinski G. 2004. Retinoblastoma protein and CCAAT/enhancer-binding protein β are required for 1,2-dihydroxyvitamin D$_3$-induced monocytic differentiation of HL60 cells. Canc Res 64:370–377.

Ji Y, Wang X, Donelly R, Uskovic M, Studzinski G. 2002. Signalling of monocytic differentiation by a hypercalcemic analog of vitamin D$_3$, 1,25(OH)$_2$-5,6-trans-16-enevitamin D$_3$, involves nuclear vitamin D receptor (nVDR) and non-nVDR-mediated pathways. J Cell Physiol 191: 198–207.

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

Kanaho Y, Nakai Y, Katoh M, Nozawa Y. 1993. The phosphatase inhibitor 2,3-diphosphoglycerate interferes with phospholipase D activation in rabbit peritoneal neutrophils. J Biol Chem 268:12492–124977.

Kang H, Lee H, Lee Y, Jo E, Kim J, Kim G, Park Y, Mindo S, Yano A, Kwak J, Bae Y. 2004. Up-regulation of phospholipase Cγ1 and phospholipase D during the differentiation of human monocytes to dendritic cells. Int immunopharmacol 4:911–920.

Kanoh H, Yamada K, Sakane F. 2002. Diacylglycerol kinases: Emerging downstream regulators in cell signalling systems. J Biochem 131:629–633.

Khare S, Bissonnette M, Wali R, Skarosi S, Boss G, Von Lintig F, Scaglione-Sewell B, Sitrin M, Brasitus T. 1999a. 1,25-dihydroxyvitamin D$_3$ but not TPA activates PLD in Caco-2 cells via pp60(c-src) and RhoA. Am J Physiol 274:G1005–G1015.

Khare S, Bissonnette M, Scaglione-Sewell B, Wali R, Sitrin M, Brasitus T. 1999b. 1,25-dihydroxyvitamin D$_3$ and TPA activate phospholipase D in Caco-2 cells: Role of PKC-α. Am J Physiol 276:G993–G1004.

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. Endocrinology 137:3649–3658.

Kim K, Kim S, Lee E, Kim N, Kang H, Kim H, Chung B, Kang C. 2001. Extracellular signal-regulated kinase/90-KDA ribosomal S6 kinase/nuclear factor-κ B pathway mediates phorbol 12-myristate 13-acetate-induced megakaryocytic differentiation of K562 cells. J Biol Chem 276:13186–13191.

Kim S, Kang S, Kim H, Kim T. 2002. Potentiation of 1,25-dihydroxyvitamin D$_3$-induced differentiation of human pro-myelocytic leukaemia cells into monocytes by costunolide, a germacranolide sesquiterpene lactone. Biochem Pharmacol 64:1233–1242.

Kolch W. 2000. Meaningful relationships: The regulation of the Ras/Raf/MEK/ERK pathway by protein interactions. Biochem J 351:289–305.

Korchak H, Corkey B, Yaney G, Kilpatrick L. 2001. Negative regulation of ligand-initiated Ca^{2+} uptake by PKC-βII in undifferentiated HL60 cells. Am J Physiol Cell Physiol 281:C514–C523.

Lal L, Li Y, Smith J, Sassano A, Uddin S, Parmar S, Tallman M, Minucci S, Hay N, Platanias L. 2005. Activation of the p70 S6 kinase by all-*trans*-retinoic acid in acute promyelocytic leukaemia cells. Blood 105:1669–1677.

Lee H, Park C, Lee Y, Suk H, Clemons T, Choi O. 2005. Antigen-induced Ca(2+) mobilization in RBL-2H3 cells: Role of I(1,4,5)P(3) and S1P and necessity of I(1,4,5)P(3) production. Cell Calc 31:581–592.

Leontieva O, Black J. 2004. Identification of two distinct pathways of protein kinase Cα down-regulation in intestinal epithelial cells. J Biol Chem 279:5788–5801.

Lewandowski D, Linassier C, Iochman S, Degenne M, Domench J, Colombat P, Binet C, Herault O. 2002. Phosphatidylinositol 3-kinases are involved in all-*trans* retinoic acid-induced upregulation of CD38 antigen on human haematopoietic cells. Brit J Haematol 118:535–544.

Li X, Alperin E, Salido E, Gong Y, Yen P, Shapiro L. 1996. Characterisation of the promoter region of human steroid sulfatase: A gene which escapes X inactivation. Somat Cell Mol Genet 22:105–117.

Liscovitch M, Czarny M, Fiucci G, Tang X. 2000. Phospholipase D: Molecular and cell biology of a novel gene family. Biochem J 345:401–415.

Lopez-Pedrera C, Dobado-Berrios P, Ros R, Torres A, Garcia-Navarro S, Jardi M, Felez J, Velasco F. 2001. Signal transduction pathways underlying the expression of tissue factor and thrombomodulin in promyelocytic cells induced to differentiate by retinoic acid and dibutyrl camp. Thromb Haemost 85:1031–1036.

Lopez-Pedrera C, Barbarroja N, Buendia P, Torres A, Dorada G, Velasco F. 2004. Promyelocytic leukemia retinoid signalling targets regulate apoptosis, tissue factor and thrombomodulin expression. Haematologica 89:286–295.

MacFarlane D, Menzel L. 1994. Activation of β-isozyme protein kinase C (PKCβ) is necessary and sufficient for phorbol ester-induced differentiation of HL: 60

promyelocytes. Studies with PKCβ-defective PET mutant. J Biol Chem 269:4327–4331.

Manfredini R, Trevisan F, Grande A, Tagliafico E, Montanari M, Lemoli R, Visana G, Tura S, Ferrari S, Ferrari S. 1999. Induction of a functional vitamin D receptor in all-trans-retinoic acid-induced monocytic differentiation of M2-type leukemic blast cells. Cancer Res 59:3803–3811.

Mansfield P, Carey S, Hinkovska-Galcheva V, Shayman J, Boxer L. 2004. Ceramide inhibition of phospholipase D and its relationship to RhoA and ARF1 translocation in GTPγS-stimulated polymorphonuclear leukocytes. Blood 103:2363–2368.

Marcinkowska E, Wiedlocha A, Radzikowski C. 1997. 1,25-dihydroxyvitamin D_3 induced activation and subsequent nuclear translocation of MAPK is upstream regulated by PKC in HL-60 cells. Biochem Biophys Res Commun 241:419–426.

Martelli A, Tazzari P, Tabelini G, Bortul R, Billi A, Manzoli L, Ruggeri A, Conte R, Cocco L. 2003. A new selective AKT pharmacological inhibitor reduces resistance to chemotherapeutic drugs, TRAIL, all-*trans*-retinoic acid, and ionising radiation of human leukaemia cells. Leukaemia 17:1794–1805.

Miguel B, Calcerrasa M, Mata F, Aller P, Clememte R, Catalan R, Martinez A. 2000. Differential redistribution of protein kinase C isoforms by cyclic AMP in HL60 cells. Biochem Biophys Res Commun 274:596–602.

Miura D, Manabe K, Gao Q, Norman A, Ishizuka S. 1999. 1a,25-dihydroxyvitamin D3-26,23-lactone analogs antagonize differentiation of human leukaemia cells (HL-60 cells) but not of human acute promyelocytic leukaemia cells (NB4 cells). FEBS Lett 460:297–302.

Morelli S, Boland R, De Boland A. 1996. 1,25$(OH)_2$-vitamin D_3 stimulation of phospholipase C and D in muscle cells involves extracellular calcium and a pertussis-sensitive G-protein. Mol Cell Endocrinol 122:207–211.

Nagashima H, Goto T. 2000. Calcium channel blockers verapamil and diltiazem impaired rubratoxin B-caused toxicity in HL60 cells. Toxicol Lett 118:47–51.

Nagpal S, Na S, Rathnalchalam R. 2005. Noncalcemic Actions of Vitamin D Receptor Ligands. Endocr Rev 26: 662–687.

Nakamura Y, Suzuki T, Sasano H. 2005. Estrogen actions and in situ synthesis in human vascular smooth muscle cells and their correlation with atherosclerosis. J Steroid Biochem Mol Biol 93:263–268.

Nakashima S, Ohguchi K, Frohman M, Nozawa Y. 1998. Increased mRNA expression of phospholipase D (PLD) isozymes during granulocytic differentiation of HL60 cells. Biochim Biophys Acta 1389:173–177.

Neri L, Bortul R, Borgatti P, Tabellini G, Baldini G, Capitani S, Martelli A. 2002. Proliferating or differentiating stimuli act on different lipid-dependent signalling pathways in nuclei of human leukaemia cells. Mol Biol Cell 13:947–964.

Neri L, Marchisio M, Colamussi M, Bertagnolo V. 1999. Monocytic differentiation of HL-60 cells is characterized by the nuclear translocation of phosphatidylinositol 3-kinase and of definite phosphatidylinositol-specfic phospholipase C isoforms. Biochem Biophys Res Commun 299:314–320.

Nguyen T-H, Lieberherr M, Fritsch J, Guillozo H, Alvarez L, Fitouri Z, Jehan F, Garabedian M. 2004. The rapid effects of 1,25$(OH)_2D_3$ require the VDR and influence 24 hydroxylase activity: Studies in human skin fibroblasts bearing vitamin D receptor point mutations. J Biol Chem 279:7591–7597.

Norman A, Olivera C, Barreto Silva F, Bishop J. 2002. A specific binding protein/receptor for 1α,25-dihydroxyvitamin D_3 is present in an intestinal caveolae membrane fraction. Biochem Biophys Res Commun 298:414–419.

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

Nussbaumer P, Billich A. 2004. Steroid sulphatase inhibitors. Med Res Rev 24:529–576.

Nutchey B, Kaplan J, Dwivedi P, Omdahl J, Ferrante A, May B, Hii C. 2005. Moleular action of 1,25-dihydroxyvitamin D_3 and phorbol ester on the activation of the rat cytochrome P450C24 (*CYP24*) promoter: Role of MAP kinase activities and identification of an Important transcription factor binding site. Biochem J 389:753–762.

Ohguchi K, Nakashima S, Tan Z, Banno Y, Dohi S, Mozawa Y. 1997. Increased activity of small GTP-binding protein-dependent phospholipase D during differentiation in human promyelocytic leukemic HL60 cells. J Biol Chem 272:1990–1996.

Pan Q, Granger J, O'Connell T, Somerman M, Simpson R. 1997. Promotion of HL-60 cell differentiation by 1,25-dihydroxyvitamin D_3 regulation of protein kinase C levels and activity. Biochem Pharmacol 54:909–915.

Pearson G, Bumeister R, Henry D, Cobb M, White M. 2000. Uncoupling Raf1 impairs only a subset of cellular responses to Raf activation. J Biol Chem 275:37303–37306.

Pongracz J, Deacon E, Johnson G, Burnett D, Lord J. 1996. Doppa induces cells death but not differentiation of U937 cells. Evidence for the involvement of PKC-β1 in the regulation of apoptosis. Leuk Res 20:319–326.

Rabano M, Pena A, Brizuela L, Macaruilla J, Gomez-Munoz A, Trueba M. 2004. Angiotensin II-stimulated cortisol secretion is mediated by phospholipase D. Mol Cell Endocrinol 222:9–20.

Racz A, Barsony J. 1999. Hormone-dependent translocation of vitamin D receptors is linked to transactivation. J Biol Chem 274:19352–19360.

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

Riechman S, Fabian T, Kroboth P, Ferrell R. 2004. Steroid sulfatase gene variation and DHEA responsiveness to resistance exercise in MERET. Physiol Genomics 17: 300–306.

Rosmarin A, Yang Z, Resendes K. 2005. Transcriptional regulation in myelopoiesis: Hematopoietic fate choice, myeloid differentiation, and leukemogenesis. Exp Hematol 33:131–143.

Rossi A, Capiati D, Picotto G, Benassati S, Boland R. 2004. MAPK inhibition by 1α,25$(OH)_2D_3$ in breast cancer cells. Evidence on the participation of the VDR and Src. J Steroid Biochem Mol Biol 89–90:278–290.

Ryves W, Dimitrijevic S, Gordge P, Evans F. 1994. HL-60 cell differentiation induced by phorbol- and 12-deoxyphorbol-esters. Carcinogenesis 15:2501–2506.

Santillan G, Katz S, Buitrago C, Boland R. 2004. 1α,25(OH)$_2$D$_3$ induces capacatative calcium entry involving a TRPC3 protein in skeletal muscle and osteoblastic cells. Biol Res 37:647–651.

Slosberg E, Yao Y, Xing F, Ikui A, Jirousck M, Weinstein I. 2000. The protein kinase Cβ-specific inhibitor blocks TPA-induced monocytic differentiation of HL60 cells. Mol Carcinog 27:166–176.

Snyder V, Turner M, Li P, El-Sharkawy L, Dunphy G, Ely D. 2000. Tissue steroid sulfatase levels, testosterone and blood pressure. J Steroid Biochem Mol Biol 73:251–256.

Song X, Bishop J, Okamura W, Norman A. 1998. Stimulation of phosphorylation of mitogen-activated protein kinase by 1α,25-dihydroxyvitamin D3 in promyelocytic NB4 leukaemia cells: A structure-function study. Endocrinology 139:457–465.

Studzinski G, Wang X, Ji Y, Wang Q, Zhang Y, Kutner A, Harrison J. 2005. The rationale for deltanoids in therapy for myeloid leukaemia: Role of KSR-MAPK-C/EBP pathway. J Steroid Biochem Mol Biol 97:47–55.

Tagoh H, Melnik S, Lefevre P, Chong S, Riggs A, Bonifer C. 2004. Dynamic reorganization of chromatin structure and selective DNA demethylation prior to stable enhancer complex formation during differentiation of primary hematopoietic cells in vitro. Blood 103:2950–2955.

Tang Q, Gromborg M, Huang H, Kim J, Otto T, Pandey A, Lane M. 2005. Sequential phosphorylation of CCAAT enhancer-binding proteinβ by MAPK and glycogen synthase kinase 3β is required for adipogenesis. Proc Natl Acad Sci USA 102:9766–9771.

Terai K, Matsuda M. 2005. Ras binding opens c-Raf to expose the docking site for mitogen-activated protein kinase kinase. EMBO Rep 6:251–256.

Toell A, Gonzalez M, Ruf D, Steinmeyer A, Carlberg C. 2001. Different molecular mechanisms of vitamin D$_3$ receptor antagonists. Mol Pharm 59:1478–1485.

Van Gompel J, Kunnimalaiyaan M, Holen K, Chen H. 2005. ZM336372, a Raf-1 activator, suppresses growth and neuroendocrine hormone levels in carcinoid tumor cells. Mol Cancer Ther 4:910–917.

Vazquez G, De Boland A, Boland R. 2000. Involvement of calmodulin in 1α,25-dihydroxyvitamin D$_3$ stimulation of store-operated Ca^{2+} influx in skeletal muscle cells. J Biol Chem 275:16134–16138.

Vermeulen L, De Wilde G, Notebaert S, Vanden Berghe W, Haegeman G. 2002. Regulation of the transcriptional activity of the nuclear factor-κB p65 subunit. Biochem Pharmacol 64:963–970.

Vertino A, Bula C, Chen J, Almeida M, Han L, Bellido T, Kousteni S, Norman A, Manolagas S. 2005. Nongenmotropic, anti-apoptotic signalling of 1α,25(OH)$_2$D$_3$ and analogs through the ligand binding domain of the vitamin D receptor in osteoblasts and osteocytes. Mediation by Src, phosphatidylinositol 2-, and JNK kinases. J Biol Chem 280:130–137.

Waldmann H, Karagunni I, Carpintero M, Gourzoulidou E, Herrmann C, Brockmann C, Oschkinat H, Muller O. 2004. Sulindac-derived Ras-pathway inhibitors target the Ras-Raf interaction and downstream effectors in the Ras pathway. Angew Chem Int Ed Engl 43:454–458.

Wang X, Studzinski G. 2001a. Activation of extracellular signal regulated kinases (ERKs) defines the first phase of 1,25-dihydroxyvitamin D$_3$-induced differentiation of HL60 cells. J Cell Biochem 80:471–482.

Wang X, Studzinski G. 2001b. Phosphorylation of Raf-1 by kinase suppessor of Ras is inhibited by "MEK-specific" inhibitors PD 098059 and U1026 in differentiating HL60 cells. Exp Cell Res 268:294–300.

Wang Q, Wang X, Studzinski G. 2003. Jun N-terminal kinase pathway enhances signalling of monocytic differentiation of human leukaemia cells induced by 1,25-dihydroxyvitamin D$_3$. J Cell Biochem 89:1087–1101.

Wang B, Gao C, Parker-Ponder K. 2005a. C/EBPβ contributes to hepatocyte growth factor-induced replication of rodent hepatocytes. J Hepatol 43:294–302.

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. 2005b. Large-scale in silico and microarray-based identification of direct 1,25-dihydroxyvitamin D$_3$ target genes. Mol Endocrinol 19:2685–2695.

Wang Q, Salman H, Danilenk M, Studzinski G. 2005c. Cooperation between antioxidants and 1,25-dihydroxyvitamin D3 in induction of leukaemia HL60 cell differentiation through the JNK/AP-1/Egr-1 pathway. J Cell Physiol 204:964–974.

Weber S, Chen J, Levitz S. 2002. Inhibition of mitogen-activated protein kinase signalling by chloroquine. J Immunol 168:5303–5309.

Yamada K, Sakane F, Imai S, Tsushima S, Murakami T, Kanoh H. 2005. Regulatory role for diacylglycerol kinase-γ in macrophage differentiation of leukaemia cells. Biochem Biophys Res Commun 305:101–107.

Yanagita T, Kobayashi H, Yamamoto R, Kataoka H, Yokoo H, Shiraishi S, Minami S, Koono M, Wada A. 2000. Protein kinase C-α and -ε down-regulate cell surface sodium channels via differential mechanisms in adrenal chromaffin cells. J Neurochem 4:1674–1684.

Yanaihara A, Yanaihara T, Toma Y, Shimuzu Y, Saito H, Okai T, Higashiya T, Osawa Y. 2001. Localization and expression of steroid sulfatase in human fallopian tubes. Steroids 66:87–91.

Yang C, Kazanietz M. 2003. Divergence and complexities in DAG signalling: Looking beyond PKC. Trends Pharm Sci 24:602–608.

Yen A, Roberson M, Varvayanis S. 1999. Retinoic acid selectively activates the ERK2 but not JNK or p38 MAP kinases when inducing myeloid differentiation. In Vitro. Cell Dev Biol Anim 35:527–532.

Zhao K-W, Li X, Zhao Q, Huang Y, Li D, Peng Z-G, Shen W-Z, Zhao J, Zhou Q, Chen Z, Sims P, Wiedmer T, Chen G-Q. 2004. Protein kinase Cδ mediates retinoic acid and phorbol nyristate aceteate-induced phospholipid scramblase I gene expression: Its role in leukaemic cell differentiation. Blood 104:3731–3738.