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The International Journal of Biochemistry & Cell Biology 38 (2006) 1181–1190
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1α,25(OH)$_2$-Vitamin D$_3$ stimulates intestinal cell p38 MAPK activity and increases c-Fos expression

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Received 1 August 2005; received in revised form 14 November 2005; accepted 21 December 2005
Available online 24 January 2006

Abstract

In intestinal cells, as in other target cells, the steroid hormone 1α,25(OH)$_2$-Vitamin D$_3$ (1α,25(OH)$_2$D$_3$) regulates gene expression via the specific intracellular Vitamin D receptor and induces fast non-transcriptional responses involving stimulation of transmembrane signal transduction pathways. We have previously shown that the hormone activates the extracellular signal-regulated mitogen-activated protein (MAP) kinase isoforms ERK1 and ERK2 in rat intestinal cells. In the present study, we have demonstrated that 1α,25(OH)$_2$D$_3$ also induces the phosphorylation and activation of p38 MAPK in these cells. The hormone effects were time and dose-dependent, with maximal stimulation at 2 min (+3-fold) and 1 nM. 1α,25(OH)$_2$D$_3$-dependent p38 phosphorylation was suppressed by SB 203580, a selective inhibitor of p38 MAPK. Ca^{2+} chelation with EGTA, inhibition of the c-Src-tyrosine kinase family with PP1 or protein kinase A (PKA) with Rp-cAMP, attenuated hormone activation of p38 MAPK. The physiological significance of 1α,25(OH)$_2$D$_3$-dependent activation of ERK1/2 and p38 MAP kinases was addressed by monitoring c-Fos expression. Incubation of intestinal cells with the hormone was followed by a rapid induction of c-Fos expression which was blocked by SB 203580 and partially suppressed by the ERK1/2 inhibitor PD 98059. Our results suggest that 1α,25(OH)$_2$D$_3$ activates p38 MAPK, involving Ca^{2+}, c-Src and PKA as upstream regulators, and that p38 MAPK has a central role in hormone-induction of the oncoprotein c-Fos in rat intestinal cells.

Keywords: Rat duodenum; 1α,25(OH)$_2$D$_3$; p38 MAPK; Ca^{2+}; PKA; c-Src; c-Fos

1. Introduction

1α,25-Dihydroxy-Vitamin D$_3$ (1α,25(OH)$_2$D$_3$) plays an essential role in the regulation of calcium homeostasis, cell proliferation and differentiation, and the immune system (Manolagas, Hustmyer, & Yu, 1990; Reichel & Norman, 1989; Walters, 1992). In rat intestinal cells (enterocytes), as in other target cells, 1α,25(OH)$_2$D$_3$ elicits responses through nuclear receptor-mediated gene transcription and a fast mechanism independent of new RNA and protein synthesis (Boland et al., 1995; Norman et al., 1992). The genomic-independent actions of 1α,25(OH)$_2$D$_3$ in enterocytes involve G protein-coupled stimulation of adenylyl cyclase and phospholipase C and activation of protein kinases A and C (Boland & Nemere, 1992). The hormone also increases intracellular Ca^{2+} levels in rat enterocytes by stimulating inner Ca^{2+} store mobilization and voltage-dependent Ca^{2+} channels through activation of second-messenger cascades (Massheimer, Boland, & de Boland, 1994; Picotto, 2001).

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doi:10.1016/j.biocel.2005.12.018

The rapid nature and specificity by which $1\alpha,25(OH)_2D_3$ activates these second messenger pathways suggest that interaction with a plasma membrane receptor is responsible for the initiation of its effects. The presence of membrane binding sites for $1\alpha,25(OH)_2D_3$ in chick intestinal cells has been described (Nemere, Dormanen, Hammond, Okamura, & Norman, 1994; Nemere et al., 2004). Alternatively other lines of evidence point to a role of the Vitamin D receptor (VDR) itself in mediating some of the rapid, non-genomic effects of the hormone (Barsony, McKoy, Renyi, & Liberman, 1994; Buitrago, Vazquez, Russo de Boland, & Boland, 2000).

Recent studies indicate that modulation of various of the fast as well as long-term responses to $1\alpha,25(OH)_2D_3$ depends on the activation of pathways implying tyrosine phosphorylation of key signalling components. In human keratinocytes the hormone rapidly stimulates Src kinase activity and Src-mediated tyrosine phosphorylation of the Shc adapter protein, which in turn associates with Grb2 and Sos (Gniadecki, 1998). Hormone-dependent Src activation has also been observed in rat colonocytes (Khare et al., 1997) and skeletal muscle cells (Buitrago et al., 2000). In intestinal cells (Boland & Norman, 1998; González Pardo & Russo de Boland, 2004), as well as in other cell types (Marcinkowska, Wiedlocha, & Radizowski, 1997; Morelli, Vazquez, Boland, & Russo de Boland, 2000; Song, Bishop, Okamura, & Norman, 1998), $1\alpha,25(OH)_2D_3$stimulates the phosphorylation and activity of the mitogen-activated protein kinases (MAPK) ERK1 and ERK2.

Five groups of MAP kinases have been identified in mammalian cells: ERK1 and ERK2, c-Jun NH_2-terminal kinase (JNK), p38 MAPK, ERK3, and Big MAPK (BMK; ERK5) (Schaeffer & Weber, 1999). Upon activation, MAPKs regulate cellular responses through the phosphorylation of other kinases, cytoplasmic and membrane proteins and transcription factors. At present it is not known whether $1\alpha,25(OH)_2D_3$ activates other members of the MAPK superfamily in intestinal cells. The present study was undertaken to examine whether $1\alpha,25(OH)_2D_3$ is able to activate p38 MAPK in its main target tissue, the duodenum.

2. Materials and methods

2.1. Materials

$1\alpha25(OH)_2D_3$ was kindly provided by Dr. Jan-Paul van de Velde from Solvay Pharmaceuticals (Weesp, The Netherlands). Phenylmethanesulphonyl fluoride (PMSF), dithiothreitol (DTT), and immobilon P (polyvinylidene difluoride, PVDF) membranes were from Sigma Chemical Co. (St. Louis, MO, USA). PP1 was from Biomol Research Lab. Inc. (Plymouth Meeting, PA). Ro 31-8220, BAPTA/AM(1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic tetra-(acetoxymethyl)ester) and Rp-cAMP (cyclic adenosine 3′,5′-monophosphorothioate, Rp diastereomer) were from Calbiochem (San Diego, CA, USA). SB 203580 and PD 98059 were from Tocris Cookson Inc. (Ellisville, MO, USA). p38 MAPK assay kit was from Cell Signaling Technology Inc. (Beverly, MA, USA). Antibodies anti-phospho p38 MAPK, anti-p38 α, anti-c-Fos and secondary antibodies donkey anti-goat and goat anti-rabbit horseradish peroxidase-conjugated IgG were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The Super Signal CL-HRP substrate system for enhanced chemiluminiscence (ECL) was obtained from Amersham Corp. (Arlington Heights, IL, USA). Other chemicals used were of analytical grade.

2.2. Animals

Young (3-month old) male Wistar rats were fed with standard rat food (1.2% calcium; 1.0% phosphorous; 4 units/g Vitamin D), given water ad libitum and maintained on a 12 h light to 12 h dark cycle. Serum levels of $1,25(OH)_2D_3$ were 67 ± 15 pg/ml. Animals were killed by cervical dislocation.

2.3. Duodenum isolation and treatment

The abdomen was opened via a midline incision, and the duodenum (10 cm from the pylorus) was exposed, excised and placed on cold physiological saline solution. The duodenum was slit lengthwise and cut into small segments (1 cm length) and pre-equilibrated for 10 min in the incubation medium containing 154 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4, 1 mM $MgCl_2$, 10 mM Na/MOPS pH 7.3, 5.6 mM glucose, 0.5% bovine serum albumin (BSA), 1 mM $CaCl_2$ (Balogh, de Boland, & Boland, 1997). After this step, the duodenum was exposed for short time-intervals (0–10 min) to $1\alpha,25(OH)_2D_3$ (0.1–100 nM) or vehicle (2-propanol 0.01%). Submerging the duodenum into liquid nitrogen ended the treatment. The mucosa was collected by scraping into ice-cold cell lysis buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin.

2.4. SDS–PAGE and Western blot analysis

Proteins were separated by one-dimensional SDS–PAGE (Laemmli, 1970). Briefly, samples were mixed with 2× Laemmli sample buffer (250 mM Tris–HCl pH 6.8, 8% SDS, 40% glycerol, 20% 2-β-mercaptoethanol and 0.02% bromophenol blue) and heated for 5 min at 95 °C. Proteins (30–35 μg) were subjected to electrophoresis on 10% SDS–polyacrylamide minigels and then transferred to PVDF membranes. The membranes were immersed in TBS–T buffer (50 mM Tris–HCl pH 7.5, 0.15 M NaCl, 0.1% Tween-20) containing 5% non-fat dry milk for 1 h to block non-specific sites. Anti-phospho-p38 MAPK (1:1000), anti-p38 α (1:1000) or anti-c-Fos (1:500) antibodies were allowed to react with the membrane overnight at 4 °C. The membranes were then washed twice (5 min) with TBS–T, followed by one 10 min wash with TBS–T and incubated 1 h with the respective secondary antibody in TBS–T 2% non-fat dry milk at room temperature. After three washes with TBS–T, bands were visualized by using an enhanced chemiluminiscent technique, according to the manufacturer's instructions. To strip the membranes for reprobing with a different antibody, the membranes were washed in stripping buffer (62.5 mM Tris–HCl, pH 6.8, 2% SDS, 50 mM 2-β-mercaptoethanol) for 30 min at 55 °C and then blocked and blotted as described above. Images were obtained with a model GS-700 imaging densitometer from Bio-Rad (Hercules, CA 94547, USA) by scanning at 600 dpi and printing at the same resolution. Bands were quantified using the Molecular Analyst program (Bio-Rad).

2.5. p38 MAPK activity assay

The activity of p38 MAPK was analyzed by using a p38 MAPK assay kit. Phosphorylated p38 was immunoprecipitated with a p38-phospho-specific antibody from 200 μg of lysate; this antibody specifically recognized phosphorylated p38 and did not cross-react with phosphorylated JNK or ERK1/2. The immune complex was washed thoroughly and resuspended in kinase buffer containing ATP and 1 μg of recombinant activating factor-2 (ATF-2) as a p38 MAPK substrate. The reaction was incubated at 30 °C for 30 min and terminated by adding SDS sample buffer. The kinase reaction was analysed by Western blotting with a phospho-specific anti-ATF-2 antibody (Banerjee, Narayanan, Mizutani, & Makino, 2002).

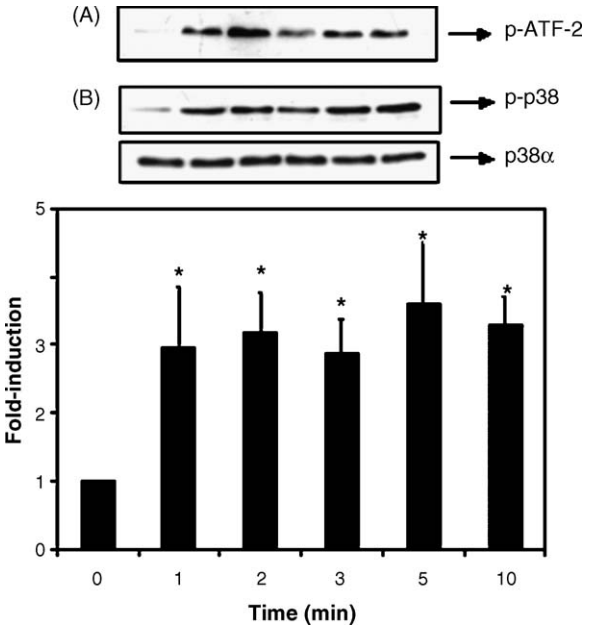

Fig. 1. Time-course of changes in p38 MAPK activity and phosphorylation in response to 1α,25(OH)$_2$D$_3$. Slices of duodenum were treated with 1 nM 1α,25(OH)$_2$D$_3$ for 0–10 min. (A) Cell lysates were immunoprecipitated with anti-phospho-p38 MAPK antibody, followed by p38 MAPK kinase assay using ATF-2 as a substrate like was described under Section 2. (B) Cell lysates were subject to SDS–PAGE electrophoresis and blotted with anti-phospho-p38 MAPK antibody. To measure the amount of total protein loaded, the membrane was reprobed with antibodies against p38α-MAPK. The immunoblots and the quantification by scanning densitometry are representative of three independent experiments; mean ± S.D. are given. $^{*}P < 0.05$.

2.6. Statistical evaluation

Statistical significance of data was evaluated using Student *t*-test (Snedecor & Cochran, 1967). Quantitative data are expressed as the mean ± S.D. from the indicated set of experiments.

3. Results

In order to evaluate if 1α,25(OH)$_2$D$_3$ stimulates p38 MAPK in intestinal cells, we investigated changes in the phosphorylation and activity of p38 MAPK. To that end, slices of duodenum were incubated with 1 nM 1α,25(OH)$_2$D$_3$ for 0–10 min. The activity of p38 kinase was assayed in vitro with activating transcription factor-2 (ATF-2) as substrate after immunoprecipitating the enzyme from cell lysates. As shown in Fig. 1A, control cells showed low or undetectable levels of activated p38; 1α,25(OH)$_2$D$_3$ caused a time-dependent increase in p38 activity, which peaked at 2 min (three-fold) and remained elevated up to 10 min. To monitor p38

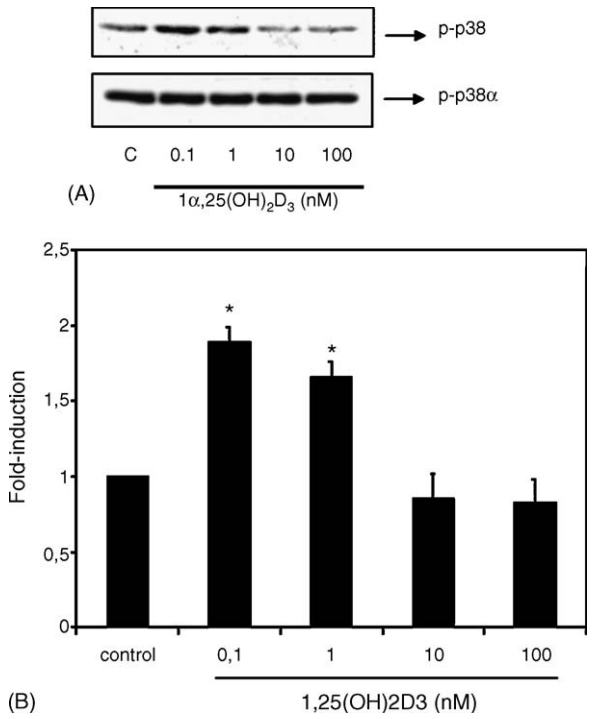

Fig. 2. Dose-response studies on $1\alpha,25(OH)_2D_3$-dependent p38 MAPK phosphorylation. Slices of duodenum were exposed to 0.1–100 nM $1\alpha,25(OH)_2D_3$ or vehicle (<0.01% 2-propanol) for 2 min. Cell extracts were analyzed by Western blotting using an anti-phospho-p38-MAPK antibody, as described in the legend of Fig. 1. (A) Representative immunoblot is shown. (B) Quantification by scanning densitometry of blots from three independent experiments; mean ± S.D. are given. $^*P < 0.05$.

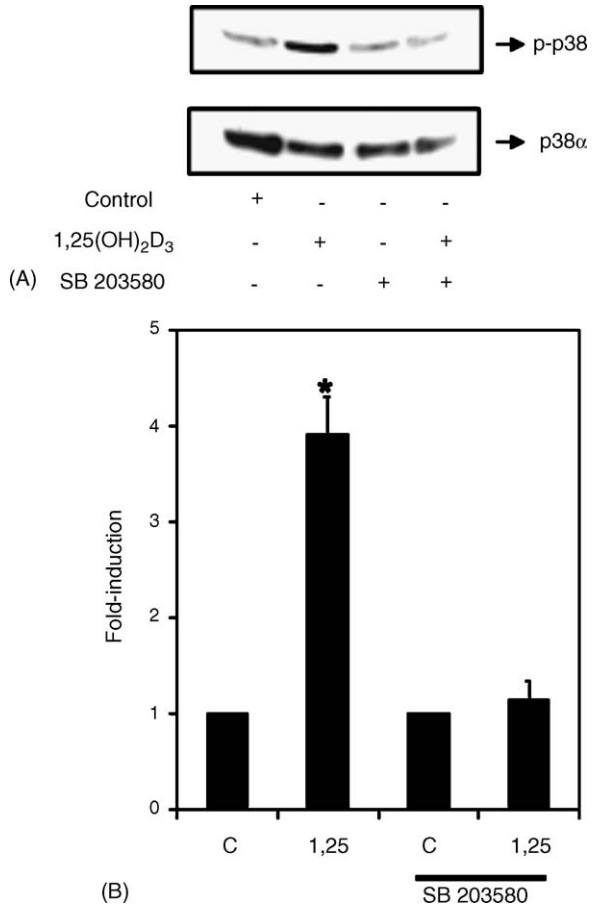

Fig. 3. $1\alpha,25(OH)_2D_3$-dependent p38 MAPK phosphorylation is blocked by SB 203580. Slices of duodenum were preincubated 10 min in the presence or absence of p38 MAPK specific pharmacological inhibitor SB 203580 (20 μM) followed by exposure to 1 nM $1\alpha,25(OH)_2D_3$ or vehicle for 2 min. Cell lysates were subjected to SDS–PAGE electrophoresis and blotted with anti-phospho-p38 MAPK antibody, membranes were striped and reprobed with p38α. (A) Representative immunoblot from three independent experiments is shown. (B) Quantification of blots from three independent experiments by scanning densitometry; mean ± S.D. are given. $^*P < 0.01$.

MAPK phosphorylation, cell lysates were subjected to SDS–PAGE and then immunoblotted with an antibody that reacts with the phosphorylated form of all p38 MAPK isoforms (α, β, γ and δ). As shown in Fig. 1B, $1\alpha,25(OH)_2D_3$ increased p38 phosphorylation, with a kinetics similar to that found for the increase in p38 kinase activity. Incubation of cells with the hormone for longer time-intervals revealed that p38 MAPK phosphorylation remained elevated up to 30 min (data not shown). The response of intestinal cells to $1\alpha,25(OH)_2D_3$ was also dose-dependent. The hormone elicited the greatest activation of p38 phosphorylation at a concentration between 0.1 and 1 nM, after a 2 min treatment interval (Fig. 2). Compound SB 203580 (20 μM), a highly specific inhibitor of P38 α/β which binds to the enzyme ATP pocket (Davis, Reddy, Caivano, & Cohen, 2000), totally blocked hormone-dependent p38 phosphorylation (Fig. 3). Two other Vitamin D_3 metabolites, $25OHD_3$ and $24,25(OH)_2D_3$, that have been shown to stimulate transcaltachia in intestinal epithelial cells (Yoshimoto & Norman, 1986; Phadnis & Nemere, 2003) were tested for their ability to activate p38 MAPK. Fig. 4 shows that both metabolites also enhanced p38 phosphorylation, and to a similar extent than $1,25(OH)_2D_3$. Contrary to Vitamin D_3 metabolites, the steroid hormone progesterone did not significantly affect p38 phosphorylation.

Since $1\alpha,25(OH)_2D_3$ increases intracellular Ca^{2+} levels in rat intestinal cells by stimulation of Ca^{2+} mobilization from inner stores and Ca^{2+} influx through voltage-dependent Ca^{2+} channels (Picotto, 2001), we evaluated whether hormone activation of p38 MAPK was mediated by calcium. Slices of duodenum were preincubated with EGTA (0.5 mM), an extracellular Ca^{2+} chelator, and the intracellular Ca^{2+} chelator BAPTA-AM (5 μM)

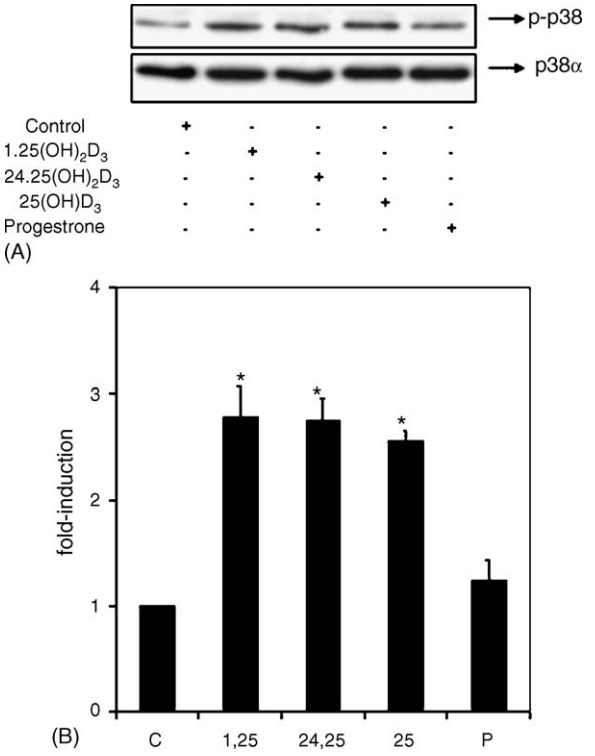

Fig. 4. Effect of 25OHD$_3$, 24,25 $(OH)_2D_3$ and progesterone on p38 MAPK phosphorylation. Slices of duodenum were exposed to 1 nM 1α,25$(OH)_2D_3$, 25OHD$_3$, 24,25$(OH)_2D_3$, progesterone or vehicle (<0.01% 2-propanol) for 2 min. Cell extracts were analyzed by Western blotting using an anti-phospho-p38-MAPK antibody, as described in the legend of Fig. 1. (A) Representative immunoblot is shown. (B) Quantification by scanning densitometry of blots from three independent experiments; mean ± S.D. are given. $^*P < 0{,}01$.

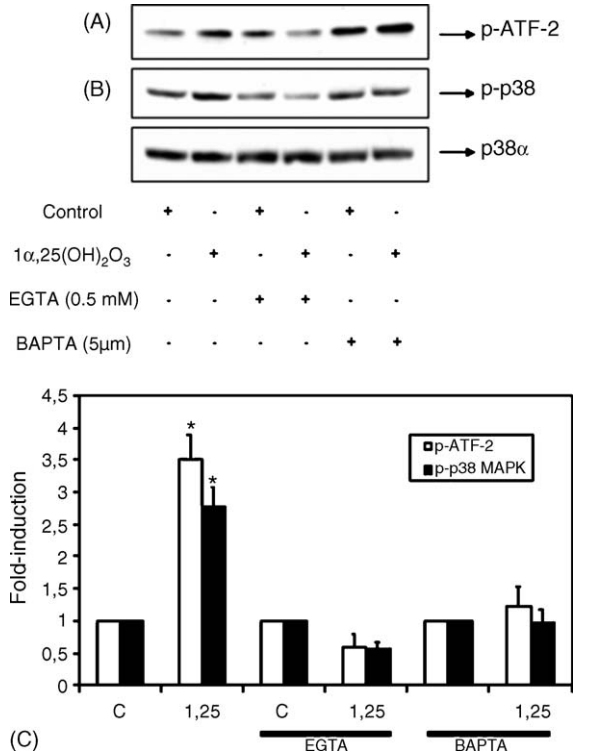

Fig. 5. Effect of calcium on 1α,25$(OH)_2D_3$-dependent p38 MAPK activity and phosphorylation. Slices of duodenum were preincubated 10 min in the presence or absence of EGTA (0.5 mM) or BAPTA-AM (5 μM) followed by exposure to 1 nM 1α,25$(OH)_2D_3$ or vehicle for 2 min. (A) Cell lysates were assayed for p38 kinase activity as described in the legend of Fig. 1. (B) Cell lysates were subjected to SDS–PAGE electrophoresis and blotted with anti-phospho-p38 MAPK antibody. Representative images and bar graphs (C) of p-p38 MAPK and p-ATF2 quantified by scanning densitometry of blots from three independent experiments are shown. Mean ± S.D. are given. $^*P < 0.05$.

followed by 2 min exposure to 1 nM 1α,25$(OH)_2D_3$ and measurement of p38 MAPK activity and phosphorylation. As shown in Fig. 5, pretreatment of rat duodenum with EGTA or BAPTA-AM inhibited the hormone-dependent changes in the phosphorylation of p38 MAPK. Treatment with the intracellular Ca^{2+} chelator alone gave values greater than basal in enterocytes, an observation which remains unexplained but consistently repeated itself throughout several experiments. These results indicate that calcium is an important mediator in the mechanism of activation of p38 MAK by 1α,25$(OH)_2D_3$.

As 1α,25$(OH)_2D_3$ increases in rat intestinal cells the intracellular levels of cAMP and activates PKA (Massheimer et al., 1994), PKC (Balogh et al., 1997) and the non-receptor tyrosine kinase c-Src (González Pardo & Russo de Boland, 2004). In the present study we examined whether these kinases are involved in hormone-stimulation of p38 MAPK. Rat duodena were preincubated with Rp-cAMP (0.2 mM), a highly specific competitive antagonist for all activators of the cAMP signal pathway (Botelho, Rothermel, Coombs, & Jastorff, 1988), the PKC inhibitor Ro 318220 (200 nM) (Davis et al., 2000) or the Src tyrosine kinase family selective inhibitor PP1 (10 μM) (Hanke et al., 1996). Duodenum slices were then treated with 1 nM 1α,25$(OH)_2D_3$ for 2 min and p38 phosphorylation assays were performed. The results shown in Fig. 6 revealed that in presence of PKA and c-Src inhibitors the phosphorylation of p38 MAPK was partially suppressed while the PKC inhibitor did not alter 1α,25$(OH)_2D_3$-induced p38 phosphorylation.

It has recently been reported that 1α,25$(OH)_2D_3$ stimulates Activator Protein DNA binding activity through an increase in c-Fos, Fra1, and c-Jun expression in human keratinocytes mediated by a mechanism dependent on phosphatidylinositol 3-kinase/Ras/MEK/ERK1/2 and c-Jun N-terminal kinase 1 (Johansen, Kragballe,

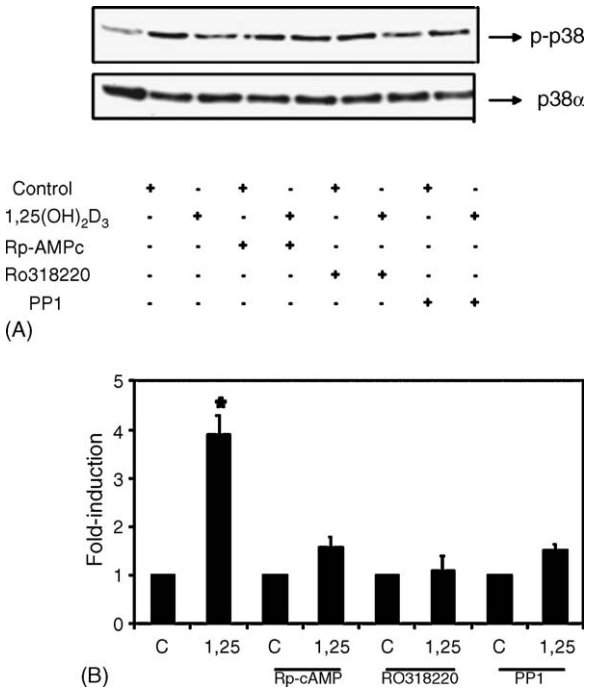

Fig. 6. Involvement of PKA, PKC and c-Src kinases in 1α,25(OH)$_2$D$_3$-dependent p38 MAPK phosphorylation. Slices of duodenum were preincubated 10 min in the presence or absence of Rp-AMPc (0.2 mM), a PKA inhibitor, PKC inhibitor Ro 318220 (200 nM) or c-Src inhibitor PP1 (10 μM), followed by exposure to 1 nM 1α,25(OH)$_2$D$_3$ or vehicle for 2 min. Cell lysates were subjected to SDS–PAGE electrophoresis and blotted with anti-phospho-p38 MAPK antibody. The immunoblot (A) and the graph (B) of quantification by scanning densitometry of three independent experiments is shown. Mean ± S.D. are given. $^{*}P < 0.05$.

Henningsen, Westergaard, & Kristiansen, 2003). This prompted us to explore whether the exposure of duodenal cells to 1α,25(OH)$_2$D$_3$ resulted in any changes in the expression of c-Jun and c-Fos proteins. To that end, whole cell extracts were prepared from duodenal cells incubated with 1 nM 1α,25(OH)$_2$D$_3$for various times. As shown in Fig. 7, c-Fos protein expression, which appeared as a band of 62 kDa, was significantly increased by 1α,25(OH)$_2$D$_3$. This effect was manifested as early as min, reached a plateau after 10 min (+2.5-fold) and returned to baseline by 20 min. c-Jun protein (39 kDa) was also detected in rat duodenal cells, but its expression was not changed by 1α,25(OH)$_2$D$_3$ (data not shown). We next examined whether 1α,25(OH)$_2$D$_3$ upregulation of the c-Fos oncoprotein synthesis in intestinal cells involves the ERK and the p38 MAP kinase pathways. To that end, rat duodenum was treated for 5 min with 1 nM 1α,25(OH)$_2$D$_3$ in the presence or absence of PD98059 (20 μM), which inhibits ERK1 and ERK2 activation by the dual MAPK kinase MEK (Alessi, Cuenda,

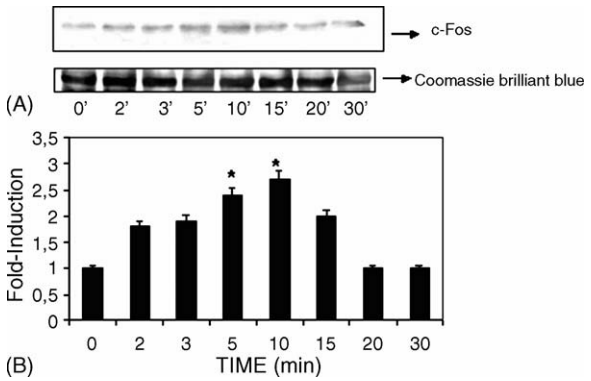

Fig. 7. Time-course of c-Fos expression induced by 1α,25(OH)$_2$D$_3$ in rat duodenum. Slices of duodenum were expossed to 1 nM 1α,25(OH)$_2$D$_3$ for 0–30 min. Cell lysates were immunoblotted with an specific antibody against c-Fos protein as described in Section 2. The membranes were dyed using Coomassie brillant blue to demonstrate equal loading. The immunoblot (A) and bar graphs (B) of c-Fos quantified by scanning densitometry of blots from three independent experiments are shown; mean ± S.D. are given. $^{*}P < 0.05$.

Cohen, Dudley, & Saltiel, 1995), and the p38 MAPK selective inhibitor, SB203580 (20 μM). Cell lysates were immunoprecipitated followed by immunobloting with a highly specific anti-c-Fos monoclonal antibody. The stimulation of the expression of c-Fos caused by 1α,25(OH)$_2$D$_3$ was partially reduced in the presence of PD98059 but completely blocked by SB203580 (Fig. 8). These results indicate that the p38 MAPK pathway, and to a lesser extent the ERK cascade, mediates 1α,25(OH)$_2$D$_3$-induced c-Fos expression in rat duodenal cells.

4. Discussion

The p38 family of mitogen-activated protein kinases are involved in regulation of cell differentiation, proliferation, apoptosis, and response to inflammation and stress (Ono & Han, 2000). p38 α, also called p38, was first cloned as a 38 kDa protein. Three other p38 isoforms (p38 β, -γ, and -δ) were isolated later and have more than 60% identity in sequence in comparison with p38 α. p38 α and p38 β are ubiquitously expressed, whereas the p38 γ and p38 δ products are only detected in certain cell types (Ono & Han, 2000). The p38 MAP kinase pathway is triggered through phosphorylation on a TGY motif within the kinase activation loop (Cano & Mahadevan, 1995). The major upstream activators of p38 MAP kinases are two MAP kinase kinases (MAPKK), MKK3 and MKK6, which directly phosphorylate the dual site in p38 MAP kinases. Once activated, p38 MAP kinases directly phosphorylate serine/threonine residues

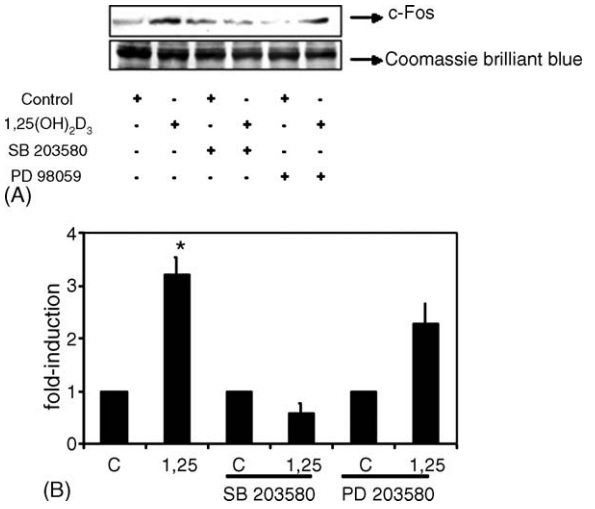

Fig. 8. Involvement of p38 MAPK and ERK1/2 in the expression of c-Fos induced by 1α,25(OH)$_2$D$_3$. Slices of duodenum were preincubated 10 min in the presence or absence of p38 inhibitor, SB 203580 (20 μM) or ERK1/2 inhibitor, PD 98059 (20 μM), followed by exposure to 1 nM 1α,25(OH)$_2$D$_3$ or vehicle for 5 min. (A) Cell lysates were immunoblotted with anti-c-Fos antibody as described in legend of Fig. 7. A representative immunoblot and quantification (B) by scanning densitometry of blots from three independent experiments are shown; mean ± S.D. are given. $^{*}P < 0.05$.

in a wide array of cytoplasmic proteins and transcription factors to mediate stress-responsive signaling (Han & Molkentin, 2000).

Little is known of the functions of p38 in intestinal epithelial cells. Recent studies have found that p38 activity appears to be involved in matrix adhesion and wound-induced signaling (Dieckgraefe, Weems, Santoro, & Alpers, 1997; Goke, Kanai, Lynch-Devaney, & Podolsky, 1998; Yu, Sanders, & Basson, 2000), whereas the p38 α isoform was identified as an important player in driving enterocyte differentiation (Houde et al., 2001). The results of the present investigation provide, to our knowledge, the first direct evidence on the stimulation of the p38 MAPK pathway by 1α,25(OH)$_2$-Vitamin D$_3$ in intestine. In our study, 1α,25(OH)$_2$D$_3$ rapidly increased p38 MAPK activity and phosphorylation in rat duodenal cells in a dose- and time-dependent fashion.

Similarly to the steroid hormone, 25OHD$_3$ and 24,25(OH)$_2$D$_3$ also enhanced p38 phosphorylation. Although the actions of these metabolites may be explained by cross-reaction with the 1,25(OH)$_2$D$_3$ membrane receptor, evidence suggest that a specific receptor also exists for 24,25(OH)$_2$D$_3$ (Nemere et al., 1994). The ability of Vitamin D$_3$ metabolites to activate p38 MAPK could also be the consequence of membrane receptor interaction with different G proteins or activation of signaling pathways.

1α,25(OH)$_2$D$_3$-induced p38 activation was inhibited by the extracellular Ca^{2+} chelator EGTA, and the intracellular Ca^{2+} chelator BAPTA-AM. In agreement with our results, Ca^{2+} has been shown to be an upstream modulator of PACAP-induced p38 MAPK activation in PC12 cells (Sakai et al., 2002). In vascular smooth muscle cells, BAPTA-AM and EGTA, also inhibited H_2O_2-stimulated p38 MAPK as well as ERK1/2 and PKB phosphorylation (Blanc, Pandey, & Srivastava, 2004). It has also been reported that p38 phosphorylation-dependent of peroxisome proliferator-activated receptor (PPAR) ligand is reduced by intracellular Ca^{2+} chelation (Gardner, Dewar, Earp, Samet, & Graves, 2003).

We investigated the signaling events underlying the effect of 1α,25(OH)$_2$D$_3$ on p38 MAP kinase activation. The role of cAMP-dependent protein kinase and Src tyrosine kinases as known upstream effectors of MAP kinases was studied. We found that in presence of chemical inhibitors of PKA and c-Src, phosphorylation of p38 MAPK was greatly diminished. Depending on the stimulus and the cell type, p38 phosphorylation has been shown to be dependent (Kabuyama, Homma, Kurosaki, & Homma, 2002; Mocsai et al., 2000; Ouwens et al., 2002) or independent (Yoshizumi, Abe, Haendeler, Huang, & Berk, 2000) of non-receptor tyrosine kinases of the Src family. Furthermore, stimulation of p38 MAPK through activation of the cAMP/PKA pathway has been demonstrated (Chio, Chang, Hsu, Chi, & Lin, 2004; Schulte & Fredholm, 2003). The partial blockade of 1α,25(OH)$_2$D$_3$-induced p38 phosphorylation by c-AMP/PKA and c-Src inhibitors, indicates that more than one signal contributes to the hormone action. The involvement of PKC on p38 phosphorylation seems to dependent on the stimulus and the cell type. Thus p38 activation has been shown to be dependent (Hofmann et al., 2004; Togo, 2004) and independent of PKC (Lemonnier, Ghayor, Guicheux, & Caverzasio, 2004). The fact that the PKC inhibitor Ro 31-81220 did not abolish the hormone-induced increase in p38 phosphorylation, rules out the participation of PKC in the action of 1α,25(OH)$_2$D$_3$ on the p38 MAPK pathway in duodenal cells. Among the p38 subfamily of MAPK, p38 α and p38 β can be specifically inhibited by SB 203580 through its binding to the ATP pocket (Gum et al., 1998) whereas p38 γ and p38 δ are not sensitive to this compound (Goedert, Cuenda, Craxton, Jakes, & Cohen, 1997). Therefore, it appears that 1α,25(OH)$_2$D$_3$ induces the phosphorylation of the p38 α and/or p38 β isoforms in intestine. Regarding the expression of individual p38 isoforms, it was reported (Vachon et al., 2002)

that distinct profiles of p38 isoform expression are exhibited by undifferentiated (α, β, γ) and differentiated (α, γ, δ) enterocytes.

P38 downstream effectors consist of kinases such as MK2 (MAPK-activating protein kinase 2) and PRAK (p38-related/activated protein kinase) and transcription factors including activating transcription factor-2 (ATF-2) and myocyte enhancement factor 2 (MEF2) (Ono & Han, 2000). In addition to these effectors, p38 can also signal through cross-talk with JNK (c-Jun NH_2-terminal kinase) (Chen, Hitomi, Han, & Stacey, 2000) and ERK pathways (Oh et al., 2000).

There is evidence of contrasting activities of the different p38 isoforms in transmitting the upstream signal to AP-1 and the expression profile of p38 isoforms determines whether the p38 signal pathway activates or inhibits AP-1-dependent processes (Pramanik et al., 2003).

In intestinal cells, we have previously demonstrated that $1\alpha,25(OH)_2D_3$ induces ERK1/2 activation (González Pardo & Russo de Boland, 2004). In other cell types, activation of ERK has been shown to increase the expression of members of the Fos protein family (Whitmarsh & Davis, 1996). In this study we found that activation of p38 MAPK and, to a lesser extent, ERK1/2, by $1\alpha,25(OH)_2D_3$ led to a rapid increase of c-Fos protein levels in duodenal cells. The hormone has been shown to increase AP-1 DNA binding activity in cultured human keratinocytes (Johansen et al., 2003) through activation of the PI3 kinase/Ras/MEK/MAPK (ERK1/2 and JNK1) signaling pathway that results in increased expression of c-Fos, Fra1, and c-Jun, that then form AP-1 dimers mediating transactivation (Johansen et al., 2003). Further studies are needed to evaluate if $1\alpha,25(OH)_2D_3$ modifies the expression of other Fos family members in intestine.

Taken together, our results indicate that $1\alpha,25(OH)_2D_3$ activates in rat duodenal cells the p38 MAPK cascade by a rapid mechanism which involves Ca^{2+}, PKA and c-Src as upstream regulators, and that p38 MAPK and ERK1/2, participate in the regulation of c-Fos expression. Since the activation of c-Fos is a common feature of the cellular response to cell-growth promoting agents, it also may play a role in the hormone regulation of intestinal cell growth and differentiation.

Acknowledgements

This research was supported by grants from the Agencia Nacional de Promoción Cientifica y Tecnológica, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) and Universidad Nacional del Sur, Argentina.

References

Alessi, D. R., Cuenda, A., Cohen, P., Dudley, D. T., & Saltiel, A. R. (1995). PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase in vitro and in vivo. *Journal of Biological Chemistry*, *270*, 27489–27494.

Balogh, G. R., de Boland, A., & Boland, R. (1997). Influence of age on $1,25(OH)_2$-Vitamin D_3 activation of protein kinase C in rat duodenum. *Molecular and Cellular Endocrynology*, *129*, 127–133.

Banerjee, S., Narayanan, K., Mizutani, T., & Makino, S. (2002). Murine coronavirus replication-induced p38 mitogen-activated protein kinase activation promotes interleukin-6 production and virus replication in cultured cells. *Journal of Virology*, *76*, 5937–5948.

Barsony, J., McKoy, W., Renyi, I., & Liberman, M. E. (1994). A common mechanism for the Vitamin D receptor activation and for the nongenomic actions of calcitriol. In A. W. Norman, R. Bouillon, & M. Thomasset (Eds.), *Vitamin D, a pluripotent hormone: Structural studies, molecular endocrinology and clinical applications* (pp. 345–346). Berlin, New York: Walter de Gruyter.

Blanc, A., Pandey, N. R., & Srivastava, A. K. (2004). Distinct roles of Ca^{2+}, calmodulin, and protein kinase C in H_2O_2-induced activation of ERK1/2, p38 MAPK, and protein kinase B signaling in vascular smooth muscle cells. *Antioxid Redox Signal*, *6*, 353–366.

Boland, A. R., & Nemere, I. (1992). Rapid actions of Vitamin D compounds. *Journal of Cellular Biochemistry*, *49*, 32–36.

de Boland, A. R., & Norman, A. W. (1998). $1\alpha,25(OH)_2$-Vitamin D_3 signaling in chick enterocytes: Enhancement of tyrosine phosphorylation and rapid stimulation of mitogen-activatrd protein (MAP) kinase. *Journal of Cellular Biochemistry*, *69*, 470–482.

Boland, R. L., de Boland, A. R., Marinissen, M., Santillan, G., Vazquez, G., & Zanello, S. (1995). Avian muscle cells as targets for the secosteroid hormone 1,25-dihydroxy-Vitamin D_3. *Molecular and Cellular Endocrinology*, *114*, 1–8.

Botelho, L. H., Rothermel, J. D., Coombs, R. V., & Jastorff, B. (1988). cAMP analog antagonists of cAMP action. *Methods Enzymology*, *159*, 159–172.

Buitrago, C., Vazquez, G., Russo de Boland, A., & Boland, R. (2000). Activation of SRC kinase in muscle cells by $1,25(OH)_2$-Vitamnin D_3 correlates with tyrosine phosphorylation of the Vitamin D receptor (VDR) and VDR–SRC interaction. *Journal of Cellular Biochemistry*, *79*, 274–281.

Buitrago, C., Vazquez, G., Boland, A. Russo de, & Boland, R. (2001). The Vitamin D receptor mediates rapid changes in muscle protein tyrosine phosphorylation induced by $1,25(OH)_2D_3$. *Biochemical Biophysical Research Communications*, *289*, 1150–1156.

Cano, E., & Mahadevan, L. C. (1995). Parallel signal processing among mammalian MAPKs. *Trends in Biochemistry. Science*, *20*, 117–122.

Chen, G., Hitomi, M., Han, J., & Stacey, D. W. (2000). The p38 pathway provides negative feedback for Ras proliferative signaling. *Journal of Biological Chemistry*, *275*, 38973–38980.

Chio, C. C., Chang, Y. H., Hsu, Y. W., Chi, K. H., & Lin, W. W. (2004). PKA-dependent activation of PKC, p38 MAPK and IKK in macrophage: Implication in the induction of inducible nitric oxide synthase and interleukin-6 by dibutyryl camp. *Cell Signal*, *16*, 565–575.

Davis, S. P., Reddy, H., Caivano, M., & Cohen, P. (2000). Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochemical Journal*, *351*, 95–105.

De Luca, H. F. (1988). The Vitamin D story: A collaborative effort of basic science and clinical medicine. *FASEB Journal*, *2*, 224–236.

Dieckgraefe, B. K., Weems, D. M., Santoro, S. A., & Alpers, D. H. (1997). ERK and p38 MAP kinase pathways are mediators of intestinal epithelial wound-induced signal transduction. *Biochemical and Biophysical Research Communications*, *233*, 389–394.

Gardner, O. S., Dewar, B. J., Earp, H. S., Samet, J. M., & Graves, L. M. (2003). Dependence of peroxisome proliferator-activated receptor ligand-induced mitogen-activated protein kinase signaling on epidermal growth factor receptor transactivation. *Journal of Biological Chemistry*, *278*, 46261–46269.

Gniadecki, R. (1998). Involvement of Src in Vitamin D signaling in human keratinocytes. *Biochemical Pharmacology*, *55*, 499–503.

Gniadecki, R. (1996). Activation of Raf-mitogen-activated protein kinase signaling pathway by 1,25-dihydroxyvitamin D_3 in normal human keratinocytes. *The Journal of Investigative Dermatology*, *106*, 1212–1217.

Goedert, M., Cuenda, A., Craxton, M., Jakes, R., & Cohen, P. (1997). Activation of the novel stress-activated protein kinase SAPK4 by cytokines and cellular stresses is mediated by SKK3 (MKK6); comparison of its substrate specificity with that of other SAP kinases. *EMBO Journal*, *16*, 3563–3571.

Goke, M., Kanai, M., Lynch-Devaney, K., & Podolsky, D. K. (1998). Rapid mitogen-activated protein kinase activation by transforming grow factor alpha in the wounded rat intestinal epithelial cells. *Gastroenterology*, *114*, 697–705.

González Pardo, V., & Russo de Boland, A. (2004). Tyrosine phosphorylation signalling dependent on 1a,25$(OH)_2$-Vitamin D_3 in rat intestinal cells: Effect of ageing. *The International Journal of Biochemistry and Cell Biology*, *36*, 489–504.

Gum, R. J., McLaughlin, M. M., Kumar, S., Wang, Z., Bower, M. J., Lee, J. C., et al. (1998). Acquisition of sensitivity of stress-activated protein kinases to the p38 inhibitor, SB 203580, by alteration of one or more amino acids within the ATP binding pocket. *Journal of Biological Chemistry*, *273*, 15605–15610.

Han, J., & Molkentin, J. D. (2000). Regulation of MEF2 by p38 MAPK and its implication in cardiomyocyte biology. *Trends in Cardiovascular Medicine*, *10*, 19–22.

Hanke, J. H., Gardner, J. P., Dow, R. L., Changelian, S., Brissette, W. H., Weringer, E. J., et al. (1996). Discovery of a novel, potent, and Src family selective tyrosine kinase inhibitor. Study of Lck- and FynT-dependent T cell activation. *Journal of Biologycal Chemistry*, *271*, 695–701.

Hofmann, M., Zaper, J., Bernd, A., Bereiter-Hahn, J., Kaufmann, R., & Kippenberger, S. (2004). Mechanical pressure-induced phosphorylation of p38 mitogen-activated protein kinase in epithelial cells via Src and protein kinase C. *Biochemical and Biophysical Research Communications*, *316*, 673–679.

Houde, M., Laprise, P., Jean, D., Blais, M., Asselin, C., & Rivard, N. (2001). Intestinal epithelial cell differentiation involves activation of p38 mitogen-activated protein kinase that regulates the homeobox trnscription factor CDX2. *Journal of Biological Biochemistry*, *276*, 21885–21894.

Johansen, C., Kragballe, K., Henningsen, J., Westergaard, M., & Kristiansen, K. (2003). 1α,25-dihydroxyvitamin D_3 stimulates activator protein 1 DNA binding activity by a phosphatidylinositol 3-kinase/Ras/MEK/extracelluar signal regulated kinase 1/2 and c-Jun N-terminal kinase 1-dependent increase in c-Fos, Fra1, and c-jun expression in human keratinocytes. *Journal of Investigative Dermatology*, *120*, 561–570.

Kabuyama, Y., Homma, M. K., Kurosaki, T., & Homma, Y. (2002). Early signaling events induced by 280 nm UV irradiation. *European Journal of Biochemistry*, *269*, 664–670.

Kakita, A., Suzuki, A., Ono, Y., Miura, Y., Itoh, M., & Oiso, Y. (2004). Possible involvement of p38 MAP kinase in prostaglandin E1-induced ALP activity in osteoblast-like cells.fata journal. *Prostaglandins Leukot Essent Fatty Acids*, *70*, 469–474.

Khare, S., Bolt, M. J. G., Wali, R. K., Skarosi, S. F., Roy, H. K., Niedziela, S., et al. (1997). 1α,25-Dihydroxyvitamin D_3 stimulates phospholipase Cg in rat colonocytes: Role of c-Src in PLCγ activation. *Journal of Clinical Investigation*, *99*, 1831–1841.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, *227*, 680–685.

Lemonnier, J., Ghayor, C., Guicheux, J., & Caverzasio, J. (2004). Protein kinase C-independent activation of protein kinase D is involved in BMP-2-induced activation of stress mitogen-activated protein kinases JNK and p38 and osteoblastic cell differentiation. *Journal of Biological Chemistry*, *279*, 259–264.

Manolagas, S. C., Hustmyer, F. G., & Yu, X.-P. (1990). Immunomodulating properties of 1,25-dihydroxyvitamin D_3. *Kidney International*, *38*, 9–16.

Marcinkowska, E., Wiedlocha, A., & Radizowski, C. (1997). 1,25-Dihydroxyvitamin D_3 induced activation and subsequent nuclear translocation of MAPK is upstream regulated by PKC in HL-60 cells. *Biochemical Biophysical Research Communications*, *241*, 419–426.

Massheimer, V., Boland, R., & de Boland, A. R. (1994). Rapid 1,25$(OH)_2$-Vitamin D_3 stimulation of calcium uptake by rat intestinal cells involves a dihydropyridine-sensitive cAMP-dependent pathway. *Cell Signal*, *6*, 299–304.

Mocsai, A., Jakus, Z., Vantus, T., Berton, G., Lowell, C. A., & Ligeti, E. (2000). Kinase pathways in chemoatractant-induced degranulation of neutrophils: The role of p38 mitogen-activated protein kinase activated by Src family kinases. *Journal of Immunology*, *164*, 4321–4331.

Morelli, S., Vazquez, G., Boland, R., & Russo de Boland, A. (2000). Involvement of tyrosine kinase activity in 1,25$(OH)_2$-Vitamnin D_3-signal transduction in skeletal muscle cells. *Journal of Biological Chemistry*, *275*, 36021–36028.

Nemere, I., Dormanen, M. C., Hammond, M. W., Okamura, W. H., & Norman, A. W. (1994). Identification of a specific binding protein for 1 alpha,25-dihydroxyvitamin D_3 in basal-lateral membranes of chick intestinal epithelium and relationship to transcaltachia. *Journal of Biological Chemistry*, *269*, 23750–23756.

Nemere, I., Farach-Carson, M. C., Rohe, B., Sterling, T. M., Norman, A. W., Boyan, B. D., et al. (2004). Ribozyme knockdown functionally links a 1,25$(OH)_2D_3$ membrane binding protein (1,25D_3-MARRS) and phosphate uptake in intestinal cells. *PNAS*, *101*, 7392–7397.

Norman, A. W., Nemere, I., Zhou, L.-X., Bishop, J. E., Lowe, K. E., Maiyar, A. C., et al. (1992). 1,25$(OH)_2$-Vitamin D_3, a steroid hormone that produces biologic effects via both genomic and nongenomic pathways. *Journal of Steroid Biochemistry and Molecular Biology*, *41*, 231–240.

Oh, C., Chang, S., Yoon, Y., Lee, S., Lee, Y., Kang, S., et al. (2000). Opposing role of mitogen-activated protein kinase subtypes, ERK1/2 and p38, in the regulation of chondrogenesis of mesenchymes. *Journal of Biological Chemistry*, *275*, 5613–5619.

Ono, K., & Han, J. (2000). The p38 signal transduction pathway activation and funcion. *Cellular Signalling*, *12*, 1–12.

Ouwens, D. M., de Ruiter, N. D., van derZon, G. C., Carter, A. P., Schouten, J., van der Burgt, C., et al. (2002). Growth factors can activate ATF2 via a two-step mechanism: Phosphorylation of Thr71 through the Ras-MEK-ERK pathway and of Thr69 through RalGDS-Src-p38. *EMBO Journal*, *21*, 3782–3793.

Phadnis, R., & Nemere, I. (2003). Direct, rapid effects of 25-hydroxyvitamin D3 on isolated intestinal cells. *Journal of Cellular Biochemistry*, *90*, 287–293.

Picotto, G. (2001). Rapid effects of calciotropic hormones on female rat enterocytes: Combined actions of $1,25(OH)_2$-Vitamin D_3, PTH and 17β-estradiol on intracellular Ca^{2+} regulation. *Hormone and Metabolic Research*, *33*, 733–738.

Pramanik, R., Qi, X., Borowicz, S., Choubey, D., Schultz, R. M., Han, J., et al. (2003). p38 Isoforms have opposite effects on AP-1-dependent transcription through regulation of c-Jun. The determinant roles of the isoforms in the p38 MAPK signal specificity. *Journal of Biological Chemistry*, *278*, 4831–4839.

Reichel, H., & Norman, A. W. (1989). Systemic effects of Vitamin D. *Annual Review of Medicine*, *40*, 71–78.

Sakai, Y., Hashimoto, H., Shintani, N., Ichibori, A., Tomimoto, S., Tanaka, K., et al. (2002). Involvement of intracellular Ca^{2+} elevation but not cyclic AMP in PACAP-induced p38 MAP kinase activation in PC12cells. *Regulatory Peptides*, *109*, 149–153.

Schaeffer, H. J., & Weber, M. J. (1999). Mitogen-activated protein kinases: Specific messages from ubiquitous messengers. *Molecular and Cellular Biology*, *19*, 2435–2444.

Schulte, G., & Fredholm, B. B. (2003). The G(s)-coupled adenosine A(2B) receptor recruits divergent pathways to regulate ERK1/2 and p38. *Experimental Cellular Research*, *290*, 168–176.

Snedecor, G. W., & Cochran, W. G. (1967). *Statistical methods*. Ames, IA: The Iowa State University Press, pp. 120–134.

Song, X., Bishop, J. E., Okamura, W. H., & Norman, A. W. (1998). Stimulation of phosphorylation of mitogen-activated protein kinase by 1α,25-dihydroxyvitamin D_3 in promyelomic NB4 leukemia cells: Estructure-funtion study. *Endocrinology*, *139*, 457–465.

Togo, T. (2004). Long-term potentiation of wound-induced exocytosis and plasma membrane repair is dependent on cAMP-response element-mediated transcription via a protein kinase C- and p38 MAPK-dependent pathway. *Journal of Biological Chemistry*, *279*, 4996–5003.

Vachon, P. H., Harnois, C., Grenier, A., Dufour, G., Bouchard, V., Han, J., et al. (2002). Differentiation State-Selective Roles of p38 Isoforms in Human Intestinal Epithelial Cell Anoikis. *Gastroenterology*, *123*, 1980–1991.

Walters, M. R. (1992). Newly identified actions of the Vitamin D endocrine system. *Endocrinology Review*, *13*, 719–764.

Whitmarsh, A. J., & Davis, R. J. (1996). Transcription factor AP-1 regulation by mitogen-activated protein kinase signal transduction pathways. *Journal of Molecular Medicine*, *74*, 589–607.

Yoshimoto, Y., & Norman, A. W. (1986). Biological activity of Vitamin D metabolites and analogs: Dose-response study of Ca^{2+} transport in an isolated chick duodenum perfusion system. *Journal of Steroid Biochemistry*, *25*, 905–909.

Yoshizumi, M., Abe, J., Haendeler, J., Huang, Q., & Berk, B. C. (2000). Src and Cas mediate JNK activation but not ERK1/2 and p38 kinases by reactive oxygen species. *Journal of Biological Chemistry*, *275*, 11706–11712.

Yu, C., Sanders, M. A., & Basson, M. D. (2000). Human Caco-2 motility redistributes FAK and paxillinin and activates p38 MAPK in a matrix dependent manner. *American Journal of Physiology*, *278*, 952–966.