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Tyrosine phosphorylation signalling dependent on 1α,25(OH)$_2$-vitamin D$_3$ in rat intestinal cells: effect of ageing

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Abstract

In intestinal cells, as in other target cells, 1α,25(OH)$_2$D$_3$ elicits long-term and short-term responses which involve genomic and non-genomic mode of actions, respectively. There is evidence indicating that activation of tyrosine phosphorylation pathways may participate in the responses induced by 1α,25(OH)$_2$D$_3$ through its non-genomic mechanism. In this study we have evaluated the involvement of 1α,25(OH)$_2$D$_3$ in the tyrosine phosphorylation of PLCγ and MAPK (ERK1/2) in enterocytes from young (3 months) and aged (24 months) rats. Immunochemical analysis revealed that the hormone stimulates PLCγ tyrosine phosphorylation in young rat enterocytes. Hormone effect on PLCγ is rapid, peaking at 2 min (+100%), is dose-dependent (10^{-10} to 10^{-8} M) and decreases with ageing. 1α,25(OH)$_2$D$_3$ also induces the phosphorylation and activation of the mitogen-activated-protein kinases ERK1 and ERK2, effect which was evident at 1 min (three-fold) and reached a maximum at 2 min (six-fold). Hormone-dependent ERK1 and ERK2 phosphorylation and activity is greatly reduced in enterocytes from old rats. In both, young and aged animals, 1α,25(OH)$_2$D$_3$-induced PLCγ and ERK1/2 phosphorylation was effectively suppressed by the tyrosine kinase inhibitor genistein (100 uM) and suppressed to a great extent by PP1, an inhibitor of c-Src kinases. LY294002, a specific inhibitor of PI3 kinase (PI3K), enzyme with an important role in mitogenesis, did not affect hormone-dependent ERK1/2 phosphorylation, indicating that PI3K is not involved in 1α,25(OH)$_2$D$_3$-induced MAPK activation. In agreement with this data, enzyme activity assays and tyrosine phosphorylation of the regulatory subunit (p85) of PI3K showed that the hormone has no effect on the enzyme activity in rat enterocytes.

Taken together, the present study suggest that in intestinal cells, tyrosine phosphorylation is an important mechanism of 1α,25(OH)$_2$D$_3$ involved in PLCγ and MAPK regulation and that this mechanism is impair with ageing.

Keywords: Rat enterocytes; 1α,25(OH)$_2$D$_3$; PLCγ; ERK1/2; Tyrosine phosphorylation; Ageing

1. Introduction

1α,25-Dihydroxy-vitamin D$_3$ (1α,25(OH)$_2$D$_3$) in addition to its classical role in the regulation of extracellular calcium homeostasis, modulates cell proliferation and differentiation and the immune system (De Luca, 1988; Manolagas, Hustmyer, & Yu, 1990; Norman, Roth, & Orci, 1982; Reichel & Norman, 1989; Walters, 1992). As in other target cells (Haussler, 1986; Boland, 1986; Lieberherr, Grosse, Duchambon, & Drueke, 1989; Minghetti & Norman, 1988; Norman et al., 1992; Boland et al., 1995),

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$1\alpha,25(OH)_2D_3$ elicits responses in rat intestinal cells (enterocytes) both through nuclear receptor-mediated gene transcription and a fast mechanism independent of new RNA and protein synthesis. The genomic-independent actions of $1\alpha,25(OH)_2D_3$ in enterocytes involve G-protein-coupled stimulation of adenylyl cyclase and phospholipase C and activation of protein kinases A and C (de 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). 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 (Nemere, Dormanen, Hammond, Okamura, & Norman, 1994) as well as for this and other steroid hormones in various cell types (reviewed in Nemere & Farach-Carson, 1999; Wehling, 1997) has been described.

Tyrosine phosphorylation is a crucial event in signal transduction mechanisms linked to the extracellular signal regulated protein kinases (MAPK) underlying the regulation of cell proliferation and differentiation by agonists of receptor tyrosine kinases or heterotrimeric G-protein-coupled receptors. Translocation of activated MAPK to the nucleus results in the phosphorylation or induction of transcription factors leading to the expression of genes involved in control of cellular growth (Marshall, 1995; Selbie & Hill, 1998). In addition, there is evidence indicating that tyrosine kinases may modulate Ca^{2+} entry both through voltage-dependent Ca^{2+} channels (Cataldi et al., 1996; Hatakeyama, Mukhopadhayay, Goyal, & Akbarali, 1996) and SOC channel (Lee, Toscas, & Villereal, 1993; Sargeant, Farndale, & Sage, 1993; Sharma & Davis, 1996) pathways.

There is evidence indicating that activation of tyrosine phosphorylation pathways participate in the responses induced by $1\alpha,25(OH)_2D_3$ through its membrane-initiated signalling. In this study we have evaluated the involvement of $1\alpha,25(OH)_2D_3$ in the tyrosine phosphorylation of PLCγ and MAPK (ERK1/2) in rat enterocytes and examined the effect of ageing on this mechanism.

2. Materials and methods

2.1. Materials

$1\alpha,25(OH)_2D_3$ was from Hoffman-La Roche (Nutley, NJ, USA). Immobilon P (Polyvinylidene difluoride, PVDF) membranes and phosphatidylinositol were from Sigma Chemical Co. (St. Louis, MO, USA). Anti-active (phospho) MAPK anti-ERK1/2 and anti-phosphotyrosine were from Promega (Madison, WI, USA). Anti-PLCγ and anti-p85α were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Secondary antibody goat anti-rabbit horseradish peroxidase-conjugated IgG and the Super Signal CL-HRP substrate system for enhanced chemiluminiscence (ECL) were obtained from Amersham Corp. (Arlington Heights, IL, USA). Other chemicals used were of analytical grade.

2.2. Animals

Young (3-month-old) and aged (24-month-old) male Wistar rats were fed with standard rat food (1.2% calcium; 1.0% phosphorous), given water ad libitum and maintained on a 12-h light:12-h dark cycle. Animals were killed by cervical dislocation.

2.3. Duodenal cell isolation

Duodenal cells were isolated as described previously (Picotto, Massheimer, & Boland, 1996). The method employed yields preparations containing only highly absorptive epithelial cells that are devoid of cells from the upper villus or crypt (Weiser, 1973). The duodenum was excised, washed with 0.9% NaCl and trimmed of adhering tissue. The intestine was slit lengthwise and cut into small segments (2 cm length) and placed into solution A containing (in mM): 96 NaCl, 1.5 KCl, 8 KH_2PO_4, 5.6 Na_2HPO_4, 27 Na citrate, pH 7.3, for 10 min at 37 °C. The solution was discarded and replaced with isolation medium containing (in mM): 154 NaCl, 10 NaH_2PO_4, 1.5 EDTA, 0.5 dithiothreitol, 5.6 glucose, pH 7.3, for 15 min at 37 °C with continuous shaking (87 oscillations/min). The cells were sedimented by centrifugation at $750 \times g$ for 10 min, washed twice with 154 mM NaCl, 10 mM NaH_2PO_4, 5.6 mM glucose, pH 7.3, and then resuspended in measurement buffer (see below). All the

steps mentioned above were performed under an atmosphere of 95% O_2:5% CO_2. Cell viability was assessed by the Trypan Blue technique. Exclusion of the dye in >90% of cells was observed for at least 90 min after isolation. Morphological characterization was performed by phase-contrast microscopy. Enterocytes isolated by this procedure have been shown to possess functional characteristics of intestinal cell (Weiser, 1973).

2.4. Immunoprecipitation

Enterocytes were treated with 1α,25$(OH)_2D_3$ and then lysed. Lysates aliquots (500–700 μg protein) were incubated overnight at 4 °C with the corresponding primary antibodies, followed by precipitation of the complexes with protein A conjugated with Sepharose. The immune complexes were washed three times with cold immunoprecipitation buffer (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 0.2 mM PMSF, 0.2 mM sodium orthovanadate, 1% Triton X-100 and 1% NP40), two times with PBS and then subjected to Western blot analysis.

2.5. 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-mercaptoethanol and 0.02% bromophenol blue) and heated for 5 min at 95 °C. Proteins (25 μg) were subjected to electrophoresis on 10% SDS–polyacrylamide minigels and then transferred to Immobilon P (PVDF) membranes. The membranes were immersed in TBS buffer (20 mM Tris–HCl pH 7.5, 150 mM NaCl) containing 5% skim milk for 2 h to block non-specific binding. Anti-active MAPK, anti-PI3K (p85α), anti-PLCγ or anti-phosphotyrosine antibodies were allowed to react with the membrane overnight at 4 °C. The membranes were then twice washed (5 min) with TBS-T (20 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.05% Tween 20), followed by one 10-min wash with TBS-T. The membranes were incubated with 1 μg/ml of goat anti-rabbit IgG antibody in TBS-T for 1 h at room temperature. After two washes with TBS-T, the membrane was visualized by using an enhanced chemiluminiscent technique (ECL, Amersham Corp.), according to the manufacturer's instructions. Images were obtained with a model GS-700 Imaging Densitometry 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).

2.6. Measurement of MAP kinase activity

Enterocytes were exposed 1 nM 1α,25$(OH)_2D_3$ for 1–10 min. Lysates were prepared followed by immunoprecipitation of MAP kinase (p42 and p44) as described above. After three washes with immunoprecipitation buffer and two washes with kinase buffer (10 mM Tris–HCl, pH 7.2, 5 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM dithiothreitol, 0.1 mM sodium orthovanadate, 1 mM phenylmethylsulphonyl fluoride, 20 μg/ml leupeptin, 20 μg/ml aprotinin and 20 μg/ml pepstatin), the immune complexes were incubated at 37 °C for 10 min in kinase buffer (50 μl/sample) containing myelin basic protein as an exogenous substrate for MAP kinase (20 μg/assay), 25 μM ATP, and [γ-^{32}P] ATP (2.5 μCi/assay). To terminate the reaction, the phosphorylated product was separated from free isotope on ion-exchange phosphocellulose filters (Whatman P-81). Papers were immersed immediately onto ice-cold 75 mM H_3PO_4, washed (1 × 5 min, 3 × 20 min) and counted in a scintillation counter.

2.7. Phosphatidylinositol 3-kinase assay

PI3K were immunoprecipitated from control or 1α,25$(OH)_2D_3$-stimulated cells (700 μg protein) using anti-phosphotyrosine. The immunoprecipitates were washed three times with ice-cold lysis buffer and then incubated in 50 μl kinase buffer (10 nM Tris–HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.1 mM Na orthovanadate) containing 0.2 mg/ml phosphatidylinositol (PI) at 37 °C for 10 min. The assay was initiated by adding 25 μCi [γ-^{32}P] ATP (10 Ci/mmol) and 20 mM $MgCl_2$, and terminated by adding 6N HCl (20 μl), and the phosphoinositol lipids were extracted with 160 μM chloroform/methanol (2:1). The phospholipid contained in the organic phase were recovered, dried, resuspended in chloroform/methanol, spotted on a silica gel 60 thin layer chromatography plates

pretreated with 1% potassium oxalate and separated in chloroform:methanol:H_2O:ammonium hydroxide (120:94:22.6:4). The phosphorylated products were visualized by autoradiography and quantified using the Molecular Analyst program (Bio-Rad).

2.8. Statistical evaluation

Statistical significance of data was evaluated using Student's *t*-test (Snedecor & Cochran, 1967). Quantitative data are expressed as the means ± S.D. from the indicated set of experiments.

3. Results

Polyphosphoinositide-PLCγ isoform is activated and associates to membranes by tyrosine phosphorylation (Hunter, 1998; Kim et al., 1991). In order to evaluate if ageing affects 1α,25$(OH)_2D_3$-dependent PLCγ tyrosine phosphorylation, enterocytes from 3- and 24-month-old rats were incubated with 1 nM 1α,25$(OH)_2D_3$ for 1–5 min and cell lysates were immunoprecipitated with anti-phosphotyrosine antibody followed by anti-PLCγ immunoblotting. As shown in Fig. 1, we found that treatment of young

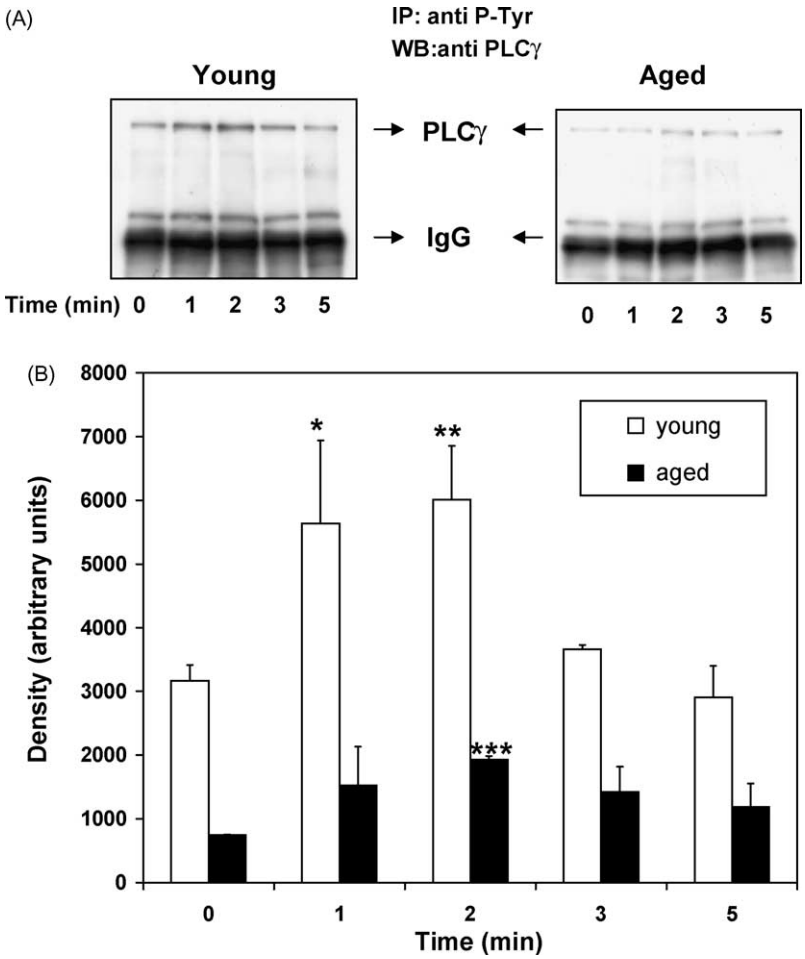

Fig. 1. Effect of age on 1α,25$(OH)_2D_3$-induced changes in rat enterocytes PLCγ tyrosine phosphorylation. Enterocytes isolated from young (3 months) and aged (24 months) rats were treated with 1 nM 1α,25$(OH)_2D_3$ 1–5 min. After cell lysis and immunoprecipitation with anti-phosphotyrosine antibody, immunoprecipitated proteins were separated by SDS–PAGE followed by Western blotting with anti-PLCγ as described in Section 2. (A) Representative immunoblots from young and aged rats are shown. (B) Quantification by scanning densitometry of blots from three independent experiments; means ± S.D. are given. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.002$.

rat duodenal cells with 1α,25(OH)$_2$D$_3$ markedly increased PLCγ-tyrosine phosphorylation. The effect was maximal between 1 and 2 min (+100%) and returned to near basal tyrosine phosphorylation levels by 5 min. In cells from aged animals, the action of the hormone was greatly diminished (+30% and +35% at 1 and 2 min, respectively). When the order of antibody addition was reversed and anti-PLCγ immunoprecipitates were probed with anti-phosphotyrosine antibody, a similar time-course of PLCγ tyrosine phosphorylation was detected (not shown). In above experiments the band corresponding to IgG heavy chains were quantified. Equal amounts of IgG were showed to be precipitated by the antibody at each of the time points. Dose–response studies with young enterocytes showed maximum effects at 1 nM, the cells from aged rats evidenced a similar profile of 1α,25(OH)$_2$D$_3$ to increase PLCγ tyrosine phosphorylation after 1 min of treatment (Fig. 2). Again, the hormone response in aged rats was markedly

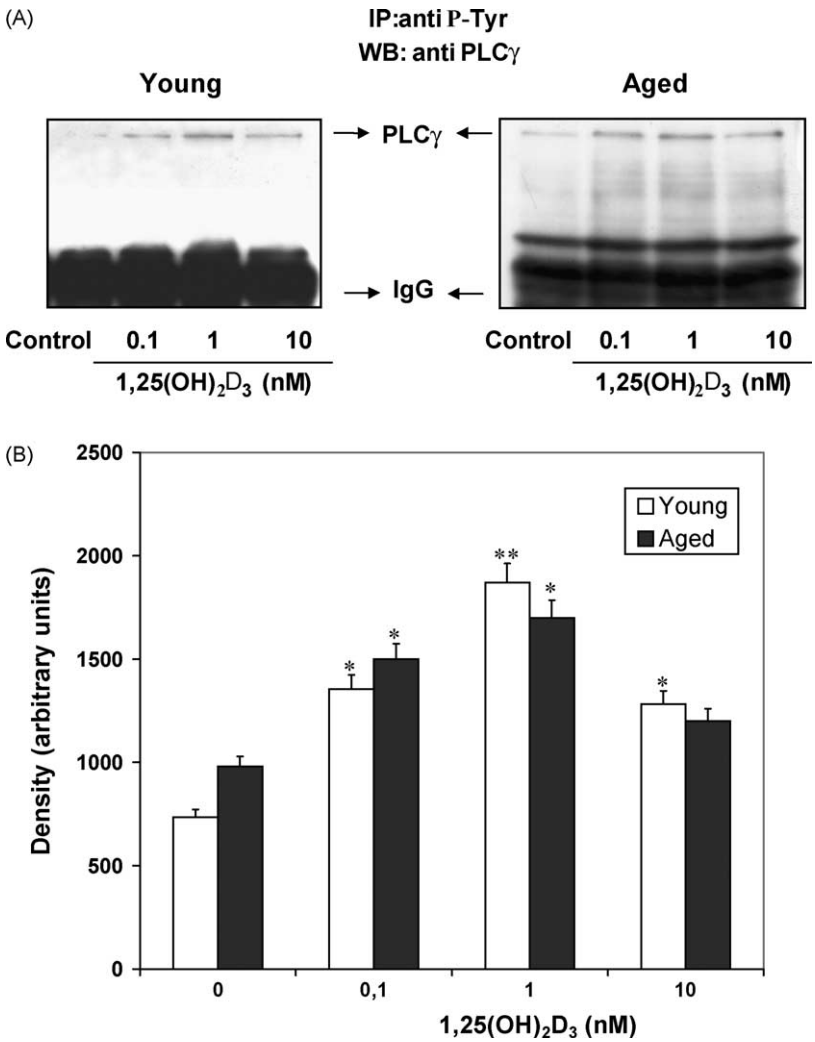

Fig. 2. Dose–response studies on 1α,25(OH)$_2$D$_3$-induced PLCγ tyrosine phosphorylation in rat enterocytes. Enterocytes isolated from young (3 months) and aged (24 months) rats were exposed for 1 min to 0.1–10 nM 1α,25(OH)$_2$D$_3$. Cell lysates were immunoprecipitated and immunoblotted as described in the legend of Fig. 1. (A) Representative immunoblots from young and aged rats are shown. (B) Quantification by scanning densitometry of blots from three independent experiments; means ± S.D. are given. $^{*}P < 0.05$; $^{**}P < 0.01$.

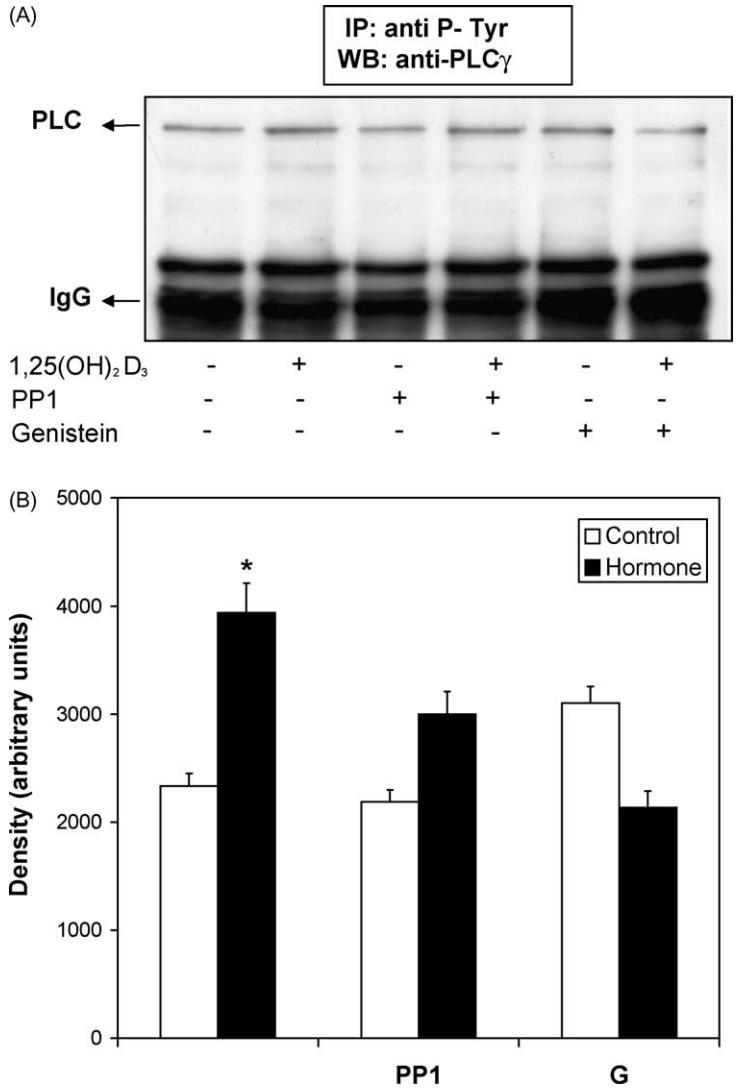

Fig. 3. Effects of PP1 and genistein on 1α,25(OH)$_2$D$_3$-induced PLCγ tyrosine phosphorylation. Enterocytes isolated from young (3 months) rats were exposed for 1 min to 1 nM 1α,25(OH)$_2$D$_3$, in the absence or presence of PP1 (10 μM) or genistein (100 μM). Cell lysates were immunoprecipitated and immunoblotted as described in the legend of Fig. 1. (A) Representative immunoblot. (B) Quantification by scanning densitometry of blots from three independent experiments; means ± S.D. are given. $^{*}P < 0.01$.

lower than in young animals at all hormone levels tested. To evaluate whether the cytosolic tyrosine kinase c-Src is part of the 1α,25(OH)$_2$D$_3$ signalling mechanism in this cells, we investigated the effect of the Src family tyrosine kinase-selective inhibitor PP1 (Hanke et al., 1996) on hormone-induced PLCγ tyrosine phosphorylation. To that end, cells from young rats were pretreated with 10 μM PP1, followed by exposure to 1 nM 1α,25(OH)$_2$D$_3$ for 1 min, under these conditions, the effects of the hormone on PLCγ phosphorylation were diminished by 50% (Fig. 3), indicating that activation of Src is part of the mechanism involved in 1α,25(OH)$_2$D$_3$ stimulation of PLCγ tyrosine phosphorylation in rat enterocytes. In addition, genistein (100 μM), a tyrosine kinases inhibitor, blocked the maximal response observed at a hormone

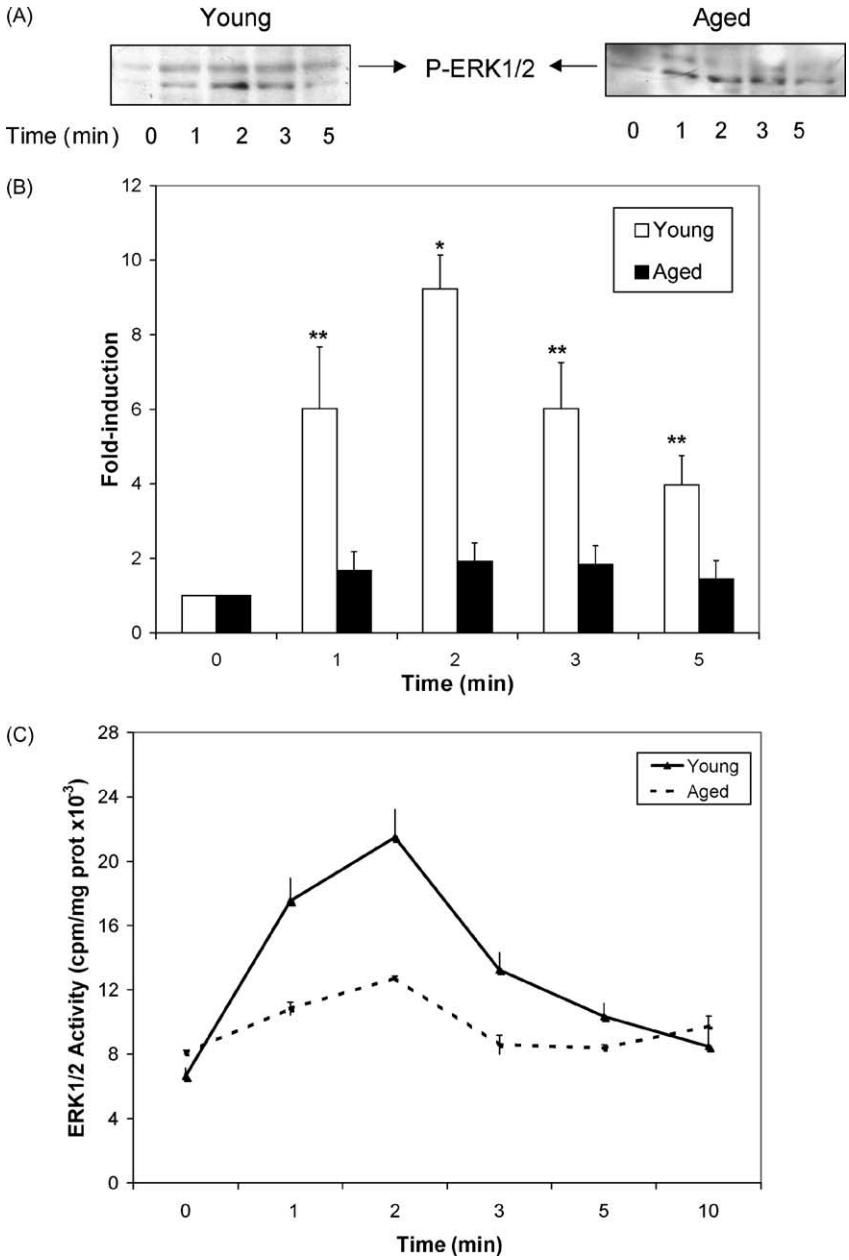

Fig. 4. Effect of age on $1\alpha,25(OH)_2D_3$-induced changes in rat enterocytes ERK1 and ERK2 tyrosine phosphorylation. Enterocytes isolated from young (3 months) and aged (24 months) rats were treated with 1 nM $1\alpha,25(OH)_2D_3$, for 1–10 min. After cell lysis, comparable aliquots of lysate proteins were separated by SDS–PAGE followed by Western blotting with anti-active (phospho) MAP kinase as described in Section 2. To measure ERK1/2 activity, cell lysates were immunoprecipitated with anti-active MAPK antibody and enzyme activity of the immunoprecipitate was measured using myelin basic protein as a substrate as described in Section 2. (A) Representative immunoblots from young and aged rats are shown. (B) Quantification by scanning densitometry of blots from three independent experiments; means ± S.D. are given. $^{*}P < 0.01$; $^{**}P < 0.05$. Bar graphs represent intensities of both phospho p42 and phospho p44 MAPK. (C) Results are the average of three independent experiments performed in duplicate ±S.D.

concentration of 1 nM (Fig. 3). Genistein per se gave values greater than basal in enterocytes, an observation which remains unexplained. One may speculate that it could be due to its interaction with enterocyte estrogen receptors and stimulation of cytosolic tyrosine kinase c-Src, which in turn, could phosphorylate PLCγ and induce phosphorylation of adaptor proteins which results in ERK phosphorylation, independently of its inhibitory action on tyrosine kinases (Di Domenico, Castoria, Bilancio, Migliaccio, & Auricchio, 1996;

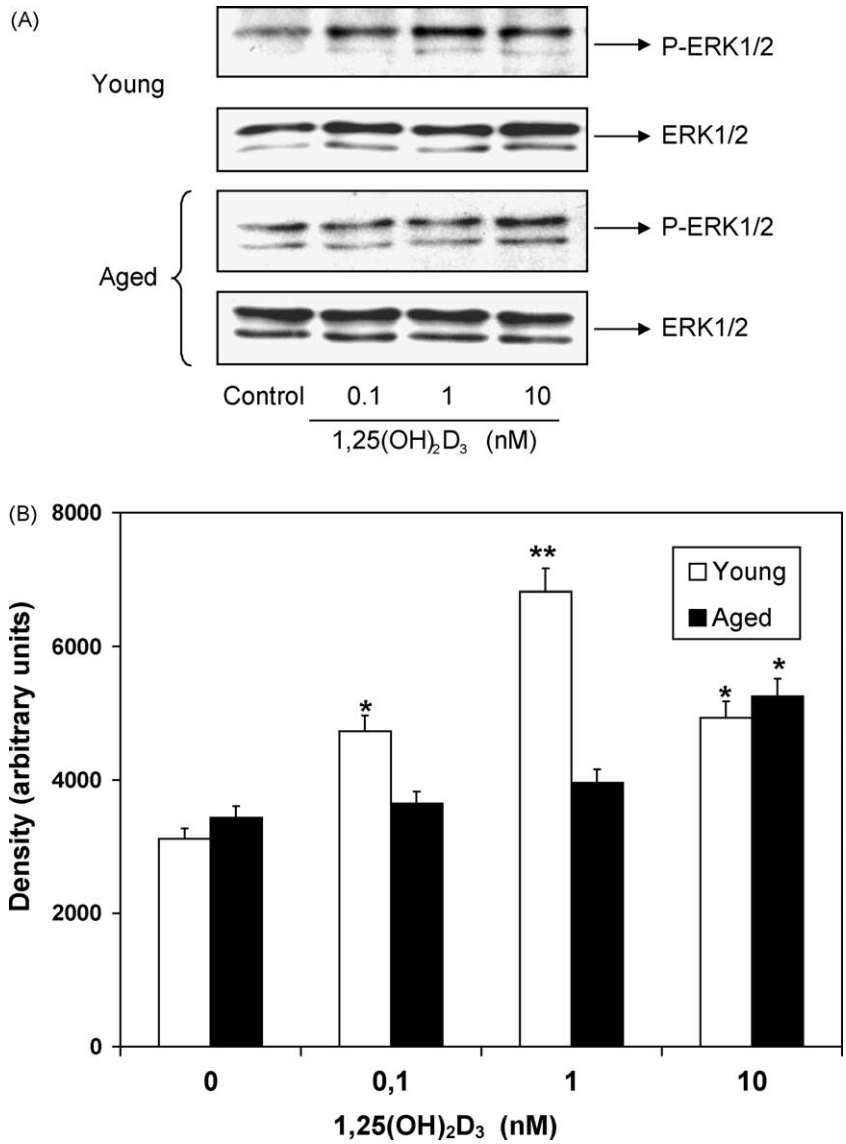

Fig. 5. Dose–response studies on 1α,25(OH)$_2$D$_3$-induced ERK1/2 tyrosine phosphorylation. Enterocytes isolated from young (3 months) and aged (24 months) rats were exposed for 1 min to 0.10–10 nM 1α,25(OH)$_2$D$_3$. After cell lysis, comparable aliquots of lysate proteins were separated by SDS–PAGE followed by Western blotting with anti-active (phospho) MAP kinase as described in Section 2. Total MAPK was measured in the same immunoblot by stripping the membrane and reincubating with anti-total MAPK (ERK1/2). (A) Representative immunoblots from young and aged rats are shown. (B) Quantification by scanning densitometry of blots from three independent experiments; means ± S.D. are given. $^{*}P < 0.05$, $^{**}P < 0.01$.

Nethrapalli et al., 2001). MAP kinase (ERKs) or extracellular signal-regulated kinase consists of 42 and 44 kDa isoforms, and requires both tyrosine and threonine phosphorylation for activation (Anderson, Maller, Tonks, & Sturgiel, 1990). To explore the possibility that $1\alpha,25(OH)_2D_3$ stimulates the phosphorylation of ERK1/2 in rat enterocytes, the cells were exposed to 1 nM $1\alpha,25(OH)_2D_3$ for 1–5 min, and proteins in lysates were separated by SDS–PAGE and then probed with anti-active MAP kinase antibody which recognizes both the 42 and 44 kDa species. Fig. 4A and B shows that the hormone significantly increased ERK1/2 tyrosine phosphorylation, with highest stimulation reached at 2 min (four-fold) decaying after 5 min of $1\alpha,25(OH)_2D_3$ exposure. Hormone-induced ERK1/2 phosphorylation was much lower (less than one-fold) in enterocytes from aged animals. To further investigate whether $1\alpha,25(OH)_2D_3$ stimulates MAP kinase activity, cells were exposed to the hormone (1–10 min) followed by immunoprecipitation of the ERK1/2 species with anti-phospho MAPK antibody and assay of immunocomplexes for kinase activity in the presence of myelin basic protein as substrate. $1\alpha,25(OH)_2D_3$ rapidly increased MAPK activity with kinetics roughly comparable with that of phosphorylation and again, the enzyme activity decreased with ageing (Fig. 4C). As shown in Fig. 5, the hormone stimulation of ERK1/2 tyrosine phosphorylation, is dose-dependent, with maximal effects achieved at 1 nM (three-fold). Again, in enterocytes from 24-month-old rats, hormone effects were less effective (less than one-fold at 10 nM). Pretreatment of

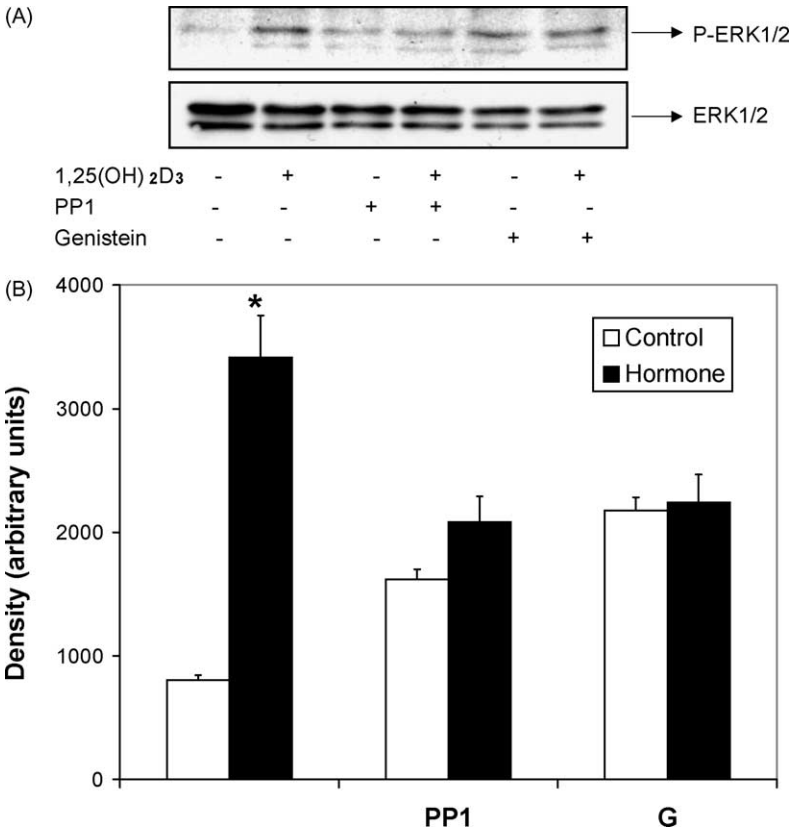

Fig. 6. Effects of genistein and PP1 on $1\alpha,25(OH)_2D_3$-induced ERK1/2 tyrosine phosphorylation. Enterocytes isolated from young (3 months) rats were exposed for 1 min to 1 nM $1\alpha,25(OH)_2D_3$, in the absence or presence of genistein (100 μM) or PP1 (10 μM). Cell lysates were immunoblotted as described in the legend of Fig. 4. Total MAPK was measured in the same immunoblot by stripping the membrane and reincubating with anti-total MAPK (ERK1/2). (A) Representative immunoblot (B) Quantification by scanning volumetric densitometry of blots from three independent experiments; means ± S.D. are given. $^*P < 0.01$.

enterocytes with the tyrosine kinase inhibitor genistein (100 μM) or the Src kinase inhibitor PP1 (10 μM) prevented 1α,25(OH)$_2$D$_3$-induced tyrosine phosphorylation of ERK1/2 (Fig. 6). PI3K has been reported to be an upstream regulator of the extracellular regulated mitogen-activated protein kinases ERK1 and ERK2 (Conway, Rakhit, Pyne, & Pyne, 1999; Cross et al., 1994; Jascur, Gilman, & Mustelin, 1997). Therefore we investigated a possible role for PI3K in the regulation of ERK1/2 by 1α,25(OH)$_2$D$_3$. To address this issue, cells were stimulated with the hormone (1 nM) in the presence of the PI3K inhibitor LY294002, and the phosphorylation of ERK1 and ERK2 was assessed by immunoblotting cell lysates with antibodies that detect the dual phosphorylated (activated) form of both ERK1 and ERK2. The results in Fig. 7, demonstrate that preincubation with LY294002 did not alter the ability of 1α,25(OH)$_2$D$_3$ to induced ERK1 and ERK2 activation. This result suggests that the PI3K pathway does not contribute to 1α,25(OH)$_2$D$_3$

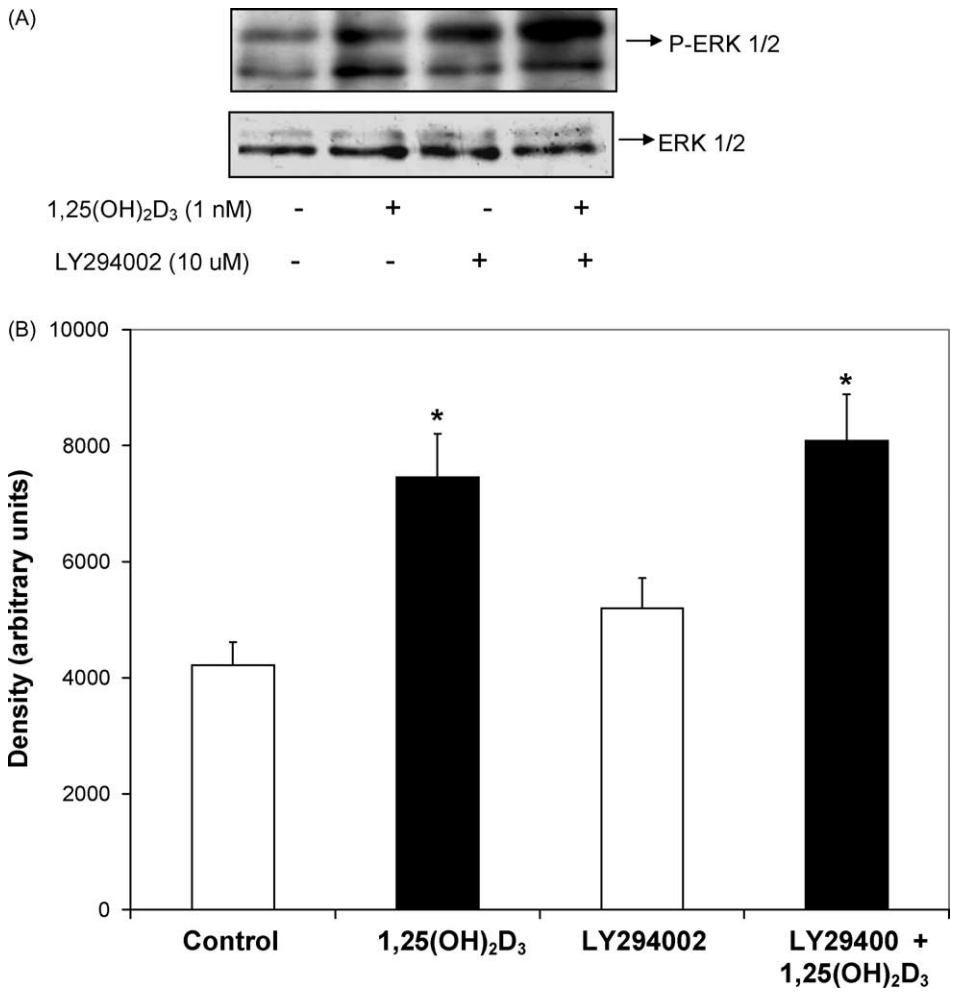

Fig. 7. Effects of PI3K inhibitor LY294002 on 1α,25(OH)$_2$D$_3$-induced ERK1/2 tyrosine phosphorylation. Enterocytes isolated from young (3 months) rats were treated with 1 nM 1α,25(OH)$_2$D$_3$ during 1 min in the presence or absence of 20 μM LY294002. The cells were then lysed and comparable aliquots of lysate proteins were separated by SDS–PAGE followed by Western blotting with anti-active (phospho) MAP kinase as described in Section 2. Total MAPK was measured in the same immunoblot by stripping the membrane and reincubating with anti-total MAPK (ERK1/2). (A) Representative immunoblot. (B) Quantification by scanning densitometry of blots from three independent experiments; means ± S.D. are given. $^*P < 0.01$.

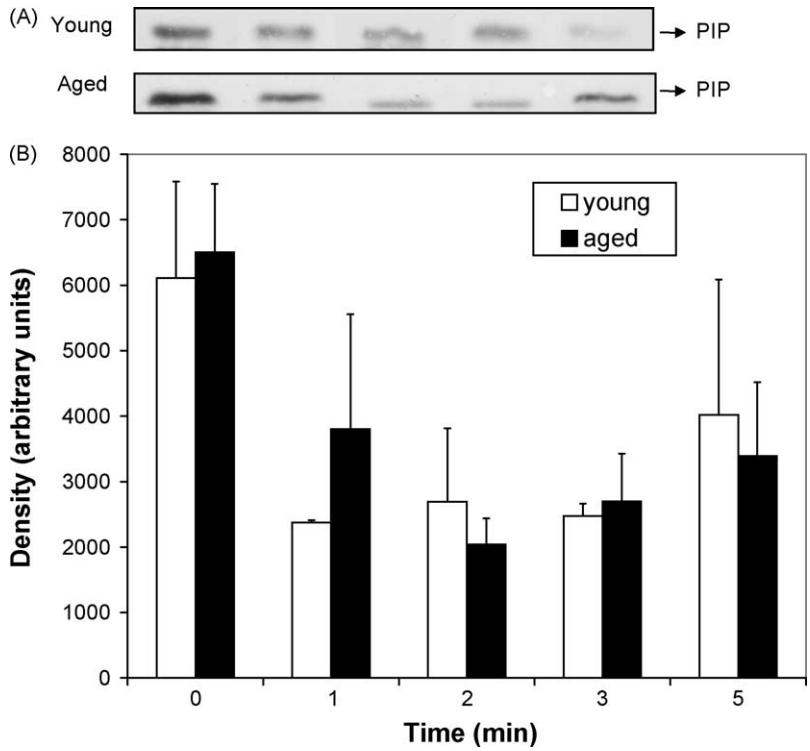

Fig. 8. 1α,25(OH)$_2$D$_3$ does not enhance PI3K activity in rat enterocytes. Cell lysates (700 μg of protein) from control and 1α,25(OH)$_2$D$_3$ (1 nM) stimulated cells, were immunoprecipitated with an antibody against P-tyrosine and immunoprecipitates were assayed for PI3K activity as described in Section 2. A representative image of the phosphorylated substrate (PIP) is shown (A). Means ± S.D. of PI3K activity is plotted as a function of the duration of hormone stimulation of cells after scanning the autoradiographic films exposed to TLC plates from three independent experiments (B). Averages ± S.D. are given.

stimulation of the MAPK signalling cascade in rat enterocytes. We then investigated if the hormone was able to stimulate PI3K activity in these cells. To that end, the enterocytes were briefly (1–5 min) stimulated with the hormone (1 nM) and PI3-kinase activity was measured after immunoprecipitation of the cell lysates with anti-phosphotyrosine. Assays with phosphatidylinositol as a substrate revealed that the lipid kinase activity of PI3K was not enhanced upon hormone treatment of either young or aged enterocytes (Fig. 8). Moreover, 1α,25(OH)$_2$D$_3$ appears to induce a fall in PIP levels, although this change is not statistically significant. These results demonstrate that the steroid hormone, at short time-intervals, which are temporally related with ERK1 and ERK2 activation, does not increase PI3K activity in rat enterocytes. In order to evaluate whether 1α,25(OH)$_2$D$_3$ affects the tyrosine phosphorylation of PI3K regulatory subunit, p85α, cells lysates obtained from either hormone-treated (1–5 min, 1 nM) enterocytes were immunoprecipitated with anti-phosphotyrosine antibody, resolved in SDS–PAGE gels and then immunoblotted with anti-p85α antibody. As shown in Fig. 9, and consistent with the above data, incubation of enterocytes, from either 3- or 24-month-old rats, with 1α,25(OH)$_2$D$_3$ does not significantly modify the level of tyrosine phosphorylation of the p85α regulatory subunit of PI3K.

4. Discussion

Responsiveness to many hormones and neurotransmitters by target tissues is altered during ageing (Roth, 1995). The Vitamin D endocrine system is involved in the reduction of intestinal calcium

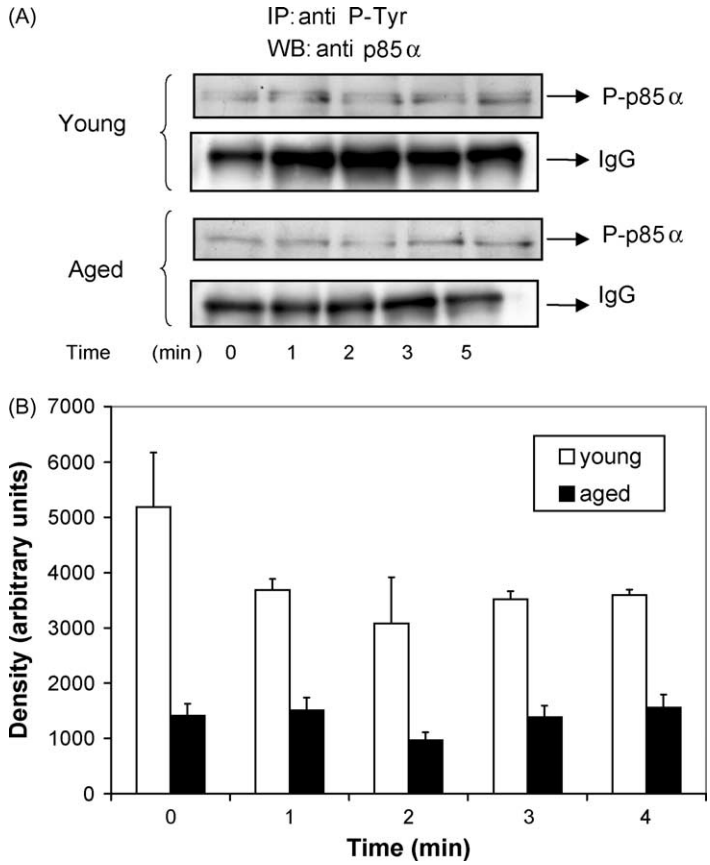

Fig. 9. 1α,25(OH)$_2$D$_3$ does not modify the tyrosine phosphorylation of p85α, the regulatory subunit of PI3K. Enterocytes isolated from young (3 months) and aged (24 months) rats were treated with 1 nM 1α,25(OH)$_2$D$_3$, for the indicated times. Cell lysates were immunoprecipitated with anti-P tyrosine, resolved on 10% SDS–PAGE gels, followed by Western blotting with anti-p85α antibody as described in Section 2. Total p85α was measured in the same immunoblot by stripping the membrane and reincubating with anti-p85α. (A) Representative immunoblot. (B) Bar graphs represent intensities of phospho-p85α quantified by scanning densitometry of blots from three independent experiments. Averages ± S.D. are given.

absorption observed with ageing (Armbrecht et al., 1989), which has been related to an impairment in both the genomic and the non-genomic modes of action of 1α,25(OH)$_2$D$_3$ (Massheimer, Picotto, de Boland, & Boland, 1995; Takamoto, Seino, Sacktor, & Liang, 1990). Age-related alterations of 1α,25(OH)$_2$D$_3$-signal transduction have been demonstrated. In enterocytes and skeletal muscle isolated from aged rats, the transient production of IP$_3$ and DAG generated by the hormone decreased significantly (de Boland, Facchinetti, Balogh, Massheimer, & Boland, 1996; Facchinetti, Boland, & de Boland, 1998). In addition, the stimulation of cAMP/protein kinase A-dependent calcium uptake by the steroid hormone in these cells is severely impaired with senescence (Massheimer et al., 1995). Moreover, age-related alterations of 1α,25(OH)$_2$D$_3$-induced duodenal PKC- and PKA-dependent protein phosphorylation has been recently reported (Balogh & de Boland, 1999). In the present study we have further examined the effects of ageing on 1α,25(OH)$_2$D$_3$-induced tyrosine phosphorylation in enterocytes isolated from 3- and 24-month-old rats. We found that the hormone stimulates, in a dose-dependent fashion, the phosphorylation of PLCγ in rat enterocytes. The response was very rapid and was suppressed by the tyrosine kinase inhibitor genistein. Immunoprecipitation and Western blot analysis demonstrated that

1α,25(OH)$_2$D$_3$-induced PLCγ tyrosine phosphorylation is greatly decreased in enterocytes from old rats. Age-related alterations in signalling events related to protein tyrosine kinase activity have been less studied. Decreased protein tyrosine phosphorylation in granulocytes and lymphocytes (Fulop, 1994) and by PTH in rat enterocytes (Gentili, Boland, & de Boland, 2001) with ageing has been reported. Two structurally related PLCγ isozymes PLCγ1 and PLCγ2 have been identified (Cockcroft & Thomas, 1992). Both isozymes have been found in association with several signalling molecules, including kinases of the Src and Syk families (Khare et al., 1997; Law, Chandran, Sidorenko, & Clark, 1996; Marrero, Schieffer, Paxton, Schieffer, & Berstein, 1995). We have recently demonstrated that 1α,25(OH)$_2$D$_3$ rapidly stimulates the enzymatic activity of c-Src tyrosine kinase in embryonic skeletal muscle cells (Buitrago, Vazquez, de Boland, & Boland, 2000), the hormone also activates c-Src in rat colonocytes (Khare et al., 1997) and human keratinocytes (Gniadecki, 1998). In further agreement with a role of tyrosine phosphorylation in 1α,25(OH)$_2$D$_3$-signalling in rat enterocytes, PP1, a specific inhibitor of the cytosolic tyrosine kinase family Src, also caused an inhibition in hormone-dependent PLCγ phosphorylation, and thus suggest that Src kinase activity participates at an upstream step of PLCγ regulated by 1α,25(OH)$_2$D$_3$ in this cells. In addition, Western analysis revealed that the hormone rapidly stimulates in rat enterocytes the tyrosine phosphorylation of the growth-related proteins MAP kinases (ERK1/2), on the basis of their immunoreactivity with corresponding selective antibody. In line with these observations, it has been reported that 1α,25(OH)$_2$D$_3$ induces a rapid stimulation of MAP kinase phosphorylation in chick enterocytes (de Boland & Norman, 1998) and in other cell types (Gniadecki, 1996; Lissoos, Beno, & Davis, 1993; Morelli, Buitrago, Vazquez, de Boland, & Boland, 2000; Song, Bishop, Okamura, & Norman, 1998). There is concordant information on the pathway by which the hormone activates MAP kinase in other cell types. It has been reported that the steroid triggers tyrosine phosphorylation of Shc and complex formation between Shc, Grb2 and Sos in keratinocytes (Gniadecki, 1996) and stimulates Raf kinase in hepatic Ito cells (Lissoos et al., 1993). Furthermore, in HL-60 promyelocytic leukemia cells it has been shown that 1α,25(OH)$_2$D$_3$ activation of MAP kinase is mediated by upstream PKC regulation (Marcinkowska, Wiedlocha, & Radizowski, 1997) and in skeletal muscle cells is mediated by PKC and Ca^{2+} (Morelli, Buitrago, Boland, & de Boland, 2001). It is not known how the MAPK cascade is regulated by 1α,25(OH)$_2$D$_3$ in rat enterocytes. Suppression of hormone effects by PP1, a specific Src family tyrosine-kinase inhibitor, indicates that 1α,25(OH)$_2$D$_3$ stimulates ERK1 and ERK2 phosphorylation via the non-receptor tyrosine kinase Src in these cells.

In addition to their lipid kinase activity, certain PI3Ks also exhibit protein kinase activity that has been implicated in the regulation of the MAPK cascade. Recent studies have shown that inhibitors of PI3K lead to inhibition of agonist-stimulated ERK1 and ERK2 activation (Conway et al., 1999; vonWillebrand et al., 1996) and different forms of dominant negative p85 mutants also inhibit agonist-stimulated ERK activation (Sharma et al., 1998; vonWillebrand et al., 1996). However, it has also been reported that PI3K inhibition does not influence ERK activity (Ferby, Waga, Hoshino, Kuma, & Shimizu, 1996; Scheid & Duronio, 1996). 1α,25(OH)$_2$D$_3$ has been shown to increase PI3K activity and differentiation in myeloid cells (Hmama et al., 1999). In rat intestinal cells the connection of PI3K to the MAPK pathway and its role in 1α,25(OH)$_2$D$_3$ signal transduction has not been explored. The fact that LY294002, a specific inhibitor of PI3K, does not alter hormone-induced ERK1 and ERK2 phosphorylation, demonstrates that PI3K activity is not required for MAPK activation in rat enterocytes stimulated with 1α,25(OH)$_2$D$_3$. In agreement with this data, enzyme activity assays and tyrosine phosphorylation of the regulatory subunit (p85α) of PI3K showed that, at short time-intervals, the hormone does not modify PI3K phosphorylation and activity in rat enterocytes. Additional experiments will be required to define the point at which signals emanating from 1α,25(OH)$_2$D$_3$ intersect with the MAPK cascade in this cells. Although the relative levels of p42 and p44 MAPK did not substantially change with age, the magnitude of 1α,25(OH)$_2$D$_3$-dependent ERK1/2 phosphorylation and activity were significantly lower in enterocytes of aged rats compared with those of young animals. Thus, our results demonstrate that ageing alters the activation of the 1α,25(OH)$_2$D$_3$-signal transduction

cascade that leads to MAPK phosphorylation and activation. Age-related decline in MAPK activation upon mitogenic stimulation has been demonstrated in hepatocytes, T lymphocytes and brain (Gorgas, Butch, & Miller, 1997; Liu et al., 1996; Palmer, Tuzon, & Paulson, 1999; Zhen, Uryu, Cai, Jhonson, & Friedman, 1999). In conclusion, the results obtained in this work expand our knowledge on the mechanism of action of $1\alpha,25(OH)_2D_3$ in rat intestine upon ageing, revealing that protein tyrosine phosphorylation is linked to $1\alpha,25(OH)_2D_3$ regulation of enterocyte PLCγ and MAPK activation, and that this mechanism is impaired with ageing and may explain age-induced alterations in the intestinal processes under the control of the hormone. Understanding the molecular mechanisms for the age-related differences in $1\alpha,25(OH)_2D_3$ signalling will require more information about the subtle mechanisms that modulate these signalling pathways.

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References

Anderson, N. G., Maller, J. L., Tonks, N. K., & Sturgiel, T. W. (1990). Requirement for integration of signals from two distinct phosphorylation pathways for activation of MAP kinase. *Nature*, *343*, 651–653.

Armbrecht, H. J., Boltz, M., Strong, R., Richardson, A., Brums, M. H. E., & Christakos, S. (1989). Expression of calbindin-D decreases with age in intestine and kidney. *Endocrinology*, *125*, 2950–2956.

Balogh, G. A., & de Boland, A. R. (1999). 1,25-Dihydroxy-vitamin D_3 (calcitriol)-dependent protein phosphorylation in rat duodenum: Effects of ageing. *Experimental Gerontology*, *34*, 983–996.

de Boland, A. R., Facchinetti, M. M., Balogh, G., Massheimer, V., & Boland, R. (1996). Age-associated decrease in inositol 1,4,5-trisphosphate and diacylglycerol generation by $1,25(OH)_2$-vitamin D_3 in rat intestine. *Cellular Signalling*, *8*, 153–157.

de 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). $1Alpha,25(OH)_2$-vitamin D_3 signaling in chick enterocytes: Enhancement of tyrosine phosphorylation and rapid stimulation of mitogen-activated protein (MAP) kinase. *Journal of Cellular Biochemistry*, *69*, 470–482.

Boland, R. L. (1986). Role of Vitamin D in skeletal muscle function. *Endocrine Review*, *7*, 434–448.

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.

Buitrago, C., Vazquez, G., de Boland, A. R., & Boland, R. (2000). Activation of Src kinase in skeletal muscle cells by 1, $1,25-(OH_2)$-vitamin D_3 correlates with tyrosine phosphorylation of the Vitamin D receptor (VDR) and VDR-Src interaction. *Journal of Cellular Biochemistry*, *79*, 274–281.

Cataldi, M., Tagliatela, M., Guerriero, S., Amoroso, S., Lombardi, G., di Renzo, G., & Annunziato, L. (1996). Protein-tyrosine kinases activate while protein-tyrosine phosphatases inhibit L-type calcium channel activity in pituitary GH3 cells. *Journal of Cellular Biochemistry*, *271*, 9441–9446.

Cockcroft, S., & Thomas, G. M. H. (1992). Inositol-lipid-specific phospholipase C isoenzymes and their differential regulation by receptors. *Biochemistry Journal*, *288*, 1–14.

Conway, A.-M., Rakhit, S., Pyne, S., & Pyne, N. J. (1999). Platelet-derived-growth- factor stimulation of the p42/p44 mitogen-activated protein kinase pathway in airway smooth muscle: Role of pertussis-toxin-sensitive G- proteins, c-Src tyrosine kinases and phosphoinositide 3-kinase. *Biochemistry Journal*, *337*, 171–177.

Cross, D. A. E., Alessi, D. R., Vandenheede, J. R., McDowell, H. E., Hundal, H. S., & Cohen, P. (1994). The inhibition of glycogen synthase kinase-3 by insulin or insulin-like growth factor 1 in the rat skeletal muscle cell line L6 is blocked by wortmannin, but not by rapamycin: Evidence that wortmannin blocks activation of the mitogen-activated protein kinase pathway in L6 cells between Ras and Raf. *Biochemistry Journal*, *303*, 21–26.

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

Di Domenico, M., Castoria, G., Bilancio, A., Migliaccio, A., & Auricchio, F. (1996). Estradiol activation of human colon carcinoma-derived Caco-2 cell growth. *Cancer Research*, *56*, 4516–4521.

Facchinetti, M. M., Boland, R., & de Boland, A. R. (1998). Age-related loss of calcitriol stimulation of phosphoinositide hydrolysis in rat skeletal muscle. *Molecular and Cellular Endocrinology*, *136*, 131–138.

Ferby, I. M., Waga, I., Hoshino, M., Kuma, K., & Shimizu, T. (1996). Wortmannin inhibits mitogen-activated protein kinase activation by platelet-activating factor through a mechanism independent of p85/p110-type phosphatidylinositol 3-kinase. *Journal of Biological Chemistry*, *271*, 11684–11688.

Fulop Jr., T. (1994). Signal transduction changes in granulocytes and lymphocytes with ageing. *Immunology Letters*, *40*, 259–268.

Gentili, C., Boland, R., & de Boland, A. R. (2001). PTH stimulates PLCbeta and PLCgamma isoenzymes in rat enterocytes: Influence of ageing. *Cellular Signalling*, *2*, 131–138.

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.

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

Gorgas, G., Butch, E. R., & Miller, R. A. (1997). Diminished activation of the MAP kinase pathway in CD_3-stimulated T lymphocytes from old mice. *Mechanism of Ageing and Development*, *94*, 71–83.

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

Hatakeyama, N., Mukhopadhayay, D., Goyal, R. K., & Akbarali, H. I. (1996). Tyrosine kinase-dependent modulation of calcium entry in rabbit colonic muscularis mucosae. *American Journal of Physiology*, *270*, C1780–C1789.

Haussler, M. R. (1986). Vitamin D receptors: Nature and function. *Annual Review of Nutrition*, *6*, 527–562.

Hmama, Z., Nandan, D., Sly, L., Knutson, K. L., Herrera-Velit, P., & Reiner, N. E. (1999). 1Alpha,25-dihydroxyvitamin D(3)-induced myeloid cell differentiation is regulated by a Vitamin D receptor-phosphatidylinositol 3-kinase signaling complex. *Journal of Experimental Medicine*, *190*, 1583–1594.

Hunter, T. (1998). The phosphorylation of proteins on tyrosine: Its role in cell growth and disease. *Philosophical Transactions of the Royal Society of London B*, *353*, 583–605.

Jascur, T., Gilman, J., & Mustelin, T. (1997). Involvement of phosphatidylinositol 3-kinase in NFAT activation in T cells. *Journal of Biological Chemistry*, *272*, 14483–14488.

Khare, S., Bolt, M. J. G., Wali, R. K., Skarosi, S. F., Roy, H. K., Niedziela, S., Scaglione-Sewell, B., Aquino, B., Abraham, C., Sitrin, M. D., Brasitus, T. A., & Bissonette, M. (1997). 1,25-Dihydroxyvitamin D_3 stimulates phospholipase C-gamma in rat colonocytes: Role of c-Src in PLC-gamma activation. *Journal of Clinical Investigation*, *99*, 1831–1841.

Kim, H. K., Kim, J. W., Zilberstein, A., Margolis, B., Kim, J. G., Schlessinger, J., & Rhee, S. G. (1991). PDGF stimulation of inositol Phospholipid hydrolysis requires PLC-gamma 1 phosphorylation on tyrosine residues 783 and 1254. *Cell*, *65*, 435–441.

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

Law, C. L., Chandran, K. A., Sidorenko, S. P., & Clark, E. (1996). A phospholipase C-gamma1 interacts with conserved phosphotyrosyl residues in the linker region of Syk and is a substrate for Syk. *Molecular and Cellular Biology*, *16*, 1305–1315.

Lee, K.-M., Toscas, K., & Villereal, M. L. (1993). Inhibition of bradykinin- and thapsigargin-induced Ca^{2+} entry by tyrosine kinase inhibitors. *Journal of Biological Chemistry*, *268*, 9945–9948.

Lieberherr, M., Grosse, B., Duchambon, P., & Drueke, R. (1989). A functional cell surface type receptor is required for the early action of 1,25-dihydroxyvitamin D_3 on the phosphoinositide metabolism in rat enterocytes. *Journal of Biological Chemistry*, *264*, 20403–20406.

Lissoos, T. W., Beno, D. W. A., & Davis, B. H. (1993). 1,25-Dihydroxyvitamin D_3 activates Raf kinase and Raf perinuclear translocation via a protein kinase C-dependent pathway. *Journal of Biological Chemistry*, *268*, 25132–25138.

Liu, Y., Guyton, K. Z., Gorospe, M., Xu, Q., Kokkonen, G. C., Mock, Y. D., Roth, G. S., & Holbrook, N. J. (1996). Age-related decline in mitogen-activated protein kinase activity in epidermal growth factor-stimulated rat hepatocytes. *Journal of Biological Chemistry*, *271*, 3604–3607.

Manolagas, S. C., Hustmyer, F. G., & Yu, X.-P. (1990). Immunomodulating properties of 1,25-dihydroxyvitamin D_3. *Kidney International*, *38*, S9–S16.

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 and Biophysical Research Communications*, *241*, 419–426.

Marrero, M. B., Schieffer, B., Paxton, W. G., Schieffer, E., & Berstein, K. E. (1995). Electroporation of pp60c-src antibodies inhibits the angiotensin II activation of phospholipase C-gamma 1 in rat aortic smooth muscle cells. *Journal of Biological Chemistry*, *270*, 15734–15738.

Marshall, C. J. (1995). Specificity of receptor tyrosine kinase signaling: Transient versus sustained extracellular signal-regulated kinase activation. *Cell*, *80*, 179–185.

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. *Cellular Signalling*, *6*, 299–304.

Massheimer, V., Picotto, G., de Boland, A. R., & Boland, R. L. (1995). Ageing alters the rapid stimulation of cAMP-dependent calcium uptake by 1,25-dihydroxy-vitamin D_3 in rat intestinal cells. *Endocrinology and Metabolism*, *2*, 157–163.

Minghetti, P. P., & Norman, A. W. (1988). 1,25$(OH)_2$-Vitamin D_3 receptors: Gene regulation and genetic circuitry. *FASEB Journal*, *2*, 3043–3053.

Morelli, S., Buitrago, C., Boland, R., & de Boland, A. R. (2001). The stimulation of MAP kinase by 1,25$(OH)_2$-vitamin D_3 in skeletal muscle cells is mediated by protein kinase C and calcium. *Molecular and Cellular Endocrinology*, *173*, 41–52.

Morelli, S., Buitrago, C., Vazquez, G., de Boland, A. R., & Boland, R. (2000). Involvement of tyrosine kinase activity in 1alpha,25$(OH)_2$-vitamin 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 1alpha,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. (1999). Membrane receptors for steroid hormones: A case for specific cell surface binding

sites for Vitamin D metabolites and estrogens. *Biochemical and Biophysical Research Communications*, *248*, 443–449.

Nethrapalli, I. S., Singh, M., Guan, X., Guo, Q., Lubahn, D. B., Korach, K. S., & Toran-Allerand, C. D. (2001). Estradiol (E_2) elicits SRC phosphorylation in the mouse neocortex: The initial event in E_2 activation of the MAPK cascade? *Endocrinology*, *142*, 5145–5148.

Norman, A. W., Nemere, Y., Zhou, L.-X., Bishop, J. E., Lowe, K. E., Maiyar, A. C., Collins, E. D., Taoka, T., Sergeev, Y., & Farach-Carson, M. C. (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.

Norman, A. W., Roth, J., & Orci, L. (1982). The Vitamin D endocrine system: Steroid metabolism, hormone receptors, and biological response. *Endocrine Reviews*, *3*, 331–365.

Palmer, H. J., Tuzon, C. T., & Paulson, K. E. (1999). Age-dependent decline in mitogenic stimulation of hepatocytes. Reduced association between Shc and the epidermal growth factor receptor is coupled to decreased activation of Raf and extracellular signal-regulated kinases. *Journal of Biological Chemistry*, *274*, 11424–11430.

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

Picotto, G., Massheimer, V., & Boland, R. (1996). Acute stimulation of intestinal cell calcium influx induced by 17 beta-estradiol via the cAMP messenger system. *Molecular and Cellular Endocrinology*, *119*, 129–134.

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

Roth, G. S. (1995). Changes in tissue responsiveness to hormones and neurotransmitters during aging. *Experimental Gerontology*, *30*, 361–368.

Sargeant, P., Farndale, R. W., & Sage, S. O. (1993). ADP- and thapsigargin-evoked Ca^{2+} entry and protein-tyrosine phosphorylation are inhibited by the tyrosine kinase inhibitors genistein and methyl-2,5-dihydroxycinnamate in fura-2-loaded human platelets. *Journal of Biological Chemistry*, *268*, 18151–18156.

Scheid, M. P., & Duronio, V. (1996). Phosphatidylinositol 3-OH kinase activity is not required for activation of mitogen-activated protein kinase by cytokines. *Journal of Biological Chemistry*, *271*, 18134–18139.

Selbie, L. A., & Hill, S. J. (1998). G protein-coupled-receptor cross-talk: The fine-tuning of multiple receptor-signalling pathways. *Trends Pharmacological Sciences*, *19*, 87–93.

Sharma, N. R., & Davis, M. J. (1996). Calcium entry activated by store depletion in coronary endothelium is promoted by tyrosine phosphorylation. *American Journal of Physiology*, *270*, H267–H274.

Sharma, P. M., Egawa, K., Huang, Y., Martin, J. L., Huvar, I., Boss, G. R., & Olefsky, J. M. (1998). Inhibition of phosphatidylinositol 3-kinase activity by adenovirus-mediated gene transfer and its effect on insulin action. *Journal of Biological Chemistry*, *273*, 18528–18537.

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

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

Takamoto, S., Seino, Y., Sacktor, B., & Liang, C. T. (1990). Effect of age on duodenal 1,25-dihydroxyvitamin D-3 receptors in Wistar rats. *Biochimica Biophysica Acta*, *1034*, 22–28.

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

Wehling, M. (1997). Specific, nongenomic actions of steroid hormones. *Annual Review of Physiology*, *59*, 365–393.

Weiser, M. (1973). Intestinal epithelial cell surface differentiation. *Journal of Biological Chemistry*, *218*, 2536–2541.

vonWillebrand, M., Jascur, T., BonnefoyBerard, N., Yano, H., Altman, A., Matsuda, Y., & Mustelin, T. (1996). Inhibition of phosphatidylinositol 3-kinase blocks T cell antigen receptor/CD_3-induced activation of the mitogen-activated kinase Erk2. *European Journal of Biochemistry*, *235*, 828–835.

Zhen, X., Uryu, K., Cai, G., Jhonson, G. P., & Friedman, E. (1999). Age-associated impairment in brain MAPK signal pathways and the effect of caloric restriction in Fischer 344 rats. *Journal of Gerontology*, *54*, B539–B548.