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1α,25(OH)$_2$-Vitamin D$_3$ Signaling in Chick Enterocytes: Enhancement of Tyrosine Phosphorylation and Rapid Stimulation of Mitogen-Activated Protein (MAP) Kinase

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Abstract The steroid hormone 1α,25(OH)$_2$–vitamin D$_3$ (1α,25(OH)$_2$D$_3$) generates biological responses in intestinal and other cells via both genomic and rapid, nongenomic signal transduction pathways. We examined the hypothesis that 1α,25(OH)$_2$D$_3$ action in chick enterocytes may be linked to pathways involving tyrosine phosphorylation. Brief exposure of isolated chick enterocytes to 1α,25(OH)$_2$D$_3$ demonstrated increased tyrosine phosphorylation of several cellular proteins (antiphosphotyrosine immunoblots of whole cell lysates) with prominent bands at 42–44, 55–60, and 105–120 Kda. The 42–44 Kda bands comigrated with mitogen-activated protein (MAP) kinase (immunoblotting with anti-MAP kinase antibody) The response occurred within 30 s, peaked at 1 min, and was dose-dependent (0.01–10 nM), with maximal stimulation at 1 nM (three- to fivefold). This effect was specific for 1α,25(OH)$_2$D$_3$ since its metabolic precursors 25(OH)D$_3$ and vitamin D$_3$ did not increase MAP kinase tyrosine phosphorylation. The tyrosine kinase inhibitor, genistein, blocked 1α,25(OH)$_2$D$_3$-induced tyrosine phosphorylation of MAP kinase, while staurosporine, a PKC inhibitor, attenuated the hormone's effects by 30%. We have evaluated the ability of 1α,25(OH)$_2$D$_3$ analogs, which have complete flexibility around the 6,7 carbon-carbon bond (6F) or which are locked in either the 6-s-*cis* (6C) or the 6-s-*trans* (6T) shape(s), to activate MAP kinase. Thus, two 6F and one 6C analog stimulated while one 6T analog did not stimulate MAP kinase tyrosine phosphorylation. In addition, 1β,25(OH)$_2$D$_3$, a known antagonist of 1α,25(OH)$_2$D$_3$-mediated rapid responses, blocked the hormone effects on MAP kinase. We conclude that 1α,25(OH)$_2$D$_3$ and analogs which can achieve the 6-s-*cis* shape (6F and 6C) can increase tyrosine phosphorylation and activation of MAP kinase in chick enterocytes. J. Cell. Biochem. 69:470–482, 1998.

Key words: enterocytes; 1,25(OH)$_2$–vitamin D$_3$; tyrosine phosphorylation; MAP kinase activation; VDR$_{nuc}$

The steroid hormone 1α,25(OH)$_2$–vitamin D$_3$ (1α,25(OH)$_2$D$_3$) plays an essential role in the maintenance of extracellular calcium homeostasis by enhancing intestinal Ca^{2+} transport [Nemere and Norman, 1991] and bone calcium mobilization [Norman and Hurwitz, 1993]. The hormone is also involved in several physiological functions unrelated to mineral homeostasis, such as immunomodulation, regulation of cell growth, and differentiation [Nemere and Norman, 1991; Bouillon et al., 1995]. Analogous to the classic steroid hormones, 1α,25(OH)$_2$D$_3$ mediates Ca^{2+} absorption in the intestine through a receptor-mediated genomic mechanism [Theofan et al., 1986; Cancela et al., 1992]. In addition, the sterol affects a variety of biological responses, in classical (intestine, bone) as well as in nonclassical target tissues, which occur independently of genome activation [Walters, 1992; de Boland and Nemere, 1992; de Boland and Boland, 1994].

1α,25(OH)$_2$D$_3$ elicits a rapid stimulation of chick intestinal Ca^{2+} transport (termed transcaltachia) [Norman, 1997] which is linked to the activation of basolateral membrane Ca^{2+} channels [de Boland et al., 1990] by protein kinase A– and protein kinase C–dependent phosphorylation [de Boland and Norman, 1990a] and also elevates the intracellular Ca^{2+}

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concentration of enterocytes [de Boland and Norman, 1990b]. Also, in rat intestinal cells the hormone increases cAMP-dependent Ca^{2+} influx through a dihydropyridine-sensitive pathway [Massheimer et al., 1994]. In addition, $1\alpha,25(OH)_2D_3$ rapidly stimulates membrane polyphosphoinositide hydrolysis and raises intracellular Ca^{2+} concentrations in both rat colonic epithelia [Wali et al., 1990] and in Caco-2 cells, a human colon cancer-derived cell line [Wali et al., 1992]. Rapid increases of the second messengers inositol trisphosphate and diacylglycerol, induced by $1\alpha,25(OH)_2D_3$ activation of phospholipase C (PLC), also have been observed in rat enterocytes [de Boland et al., 1996] as well as in various other cell types [de Boland and Nemere, 1992; Bourdeau et al., 1990; Morelli et al., 1993]. In intestinal cells [Wali et al., 1990; Marinissen et al., 1994] and in other cells [Marinissen et al., 1994; Bissonette et al., 1994; Simboli-Campbell et al., 1994; Gniadecki, 1994], the hormone rapidly stimulates protein kinase C, an enzyme that is thought to play a crucial role in the regulation of the cell cycle and modulation of ion channels [Nishizuka, 1992].

The rapid response signal transduction components of the $1\alpha,25(OH)_2D_3$ cascade have been postulated to be involved in the regulation of cell growth [Gniadecki, 1994; Yada et al., 1989]. In this regard, activation of mitogen-activated protein kinases (MAPKs) has been shown to be essential for stimulation of growth in many cells [Pelicci et al., 1992; Blenis, 1993; Laird et al., 1995]. Recently $1\alpha,25(OH)_2D_3$ activation of Raf-MAP kinase pathways has been reported in hepatocytes [Beno et al., 1995] and keratinocytes [Gniadecki, 1996]. Also, estrogen has been recently reported to effect a rapid activation of MAP kinase in ROS17/2.8 cells [Endoh et al., 1997].

One characteristic property of vitamin D seco steroids[1] is that they can undergo rotation about the 6,7 carbon-carbon bond which permits generation of a continuum of potential ligand shapes, extending from the 6-s-*cis* (steroid-like conformation) to the 6-s-*trans* (extended steroid conformation; see Fig. 5A). We have previously presented evidence that is consistent with the model that the genomic and nongenomic/rapid responses have distinct preferences with regard to the conformation of their agonist ligand [Norman et al., 1997].

In the present study, we have further investigated $1\alpha,25(OH)_2D_3$ signal transduction pathways in chick enterocytes. We have examined the hypothesis that conformationally specific shapes of the secosteroid hormone may be linked to responses involving tyrosine phosphorylation which result in the activation of MAP kinase that in turn may participate in the hormone's mitogenic rapid actions in this target organ.

MATERIALS AND METHODS

Chemicals

$1\alpha,25(OH)_2D_3$, $25(OH)D_3$, vitamin D_3, and analog AT were kindly donated by Dr. M.R. Uskokovic (Hoffmann-LaRoche, Nutley, NJ); analog BT is the kind gift of Leo Pharmaceuticals (Ballerup, Denmark); $1\alpha,25(OH)_2D_3$ analogs were generously provided by Dr. W.H. Okamura (Riverside, CA). Rabbit polyclonal antiphosphotyrosine antibody was obtained from Upstate Biotechnology Inc. (Lake Placid, NY); antiactive MAP kinase antibody (reactive against p42 and p44 isoforms) was obtained from Promega (Madison, WI); secondary antibody goat antirabbit horseradish peroxidase–conjugated IgG, the Super Signal CL-HRP substrate system for enhanced chemiluminiscence (ECL), and $\gamma^{32}P$ ATP (3,000 Ci/mmol) were from Amersham Corp. (Arlington Heights, IL); and protein A-Sepharose was purchased from Pierce Chemical Co. (Rockford, IL). All other reagents were from Sigma Chemical Co. (St Louis, MO).

Enterocyte Isolation and Treatment

Duodenal cells were isolated from 3–4-week-old White Leghorn chicks essentially as previously described [Walters and Weiser, 1987]. The duodenum was excised, washed with 0.9% NaCl, and trimmed of adhering tissue. The intestine was slit lengthwise and placed into solution A (96 mM NaCl, 1.5 mM KCl, 8 mM KH_2PO_4, 5.6 mM Na_2HPO_4, 27 mM Na citrate, pH 7.3) for 10 min at room temperature with mild shaking. The solution was discarded and replaced with solution B (isolation medium; 154 mM NaCl, 10 mM NaH_2PO_4, 0.5 mM EDTA, 0.5 mM dithio-

[1]Seco steroids are by definition compounds in which one of the cyclopentanoperhydrophenanthrene rings of the steroid ring structure is broken. In the case of vitamin D_3 the 9,10 carbon-carbon bond of the B ring is broken generating a seco-B steroid. See Figure 5A for examples. The official IUPAC name for vitamin D_3 is 9,10-secocholesta-5,7,10(19)-trien-3b-ol.

threitol, 5.6 mM glucose, pH 7.3) for 15 min at room temperature with vigorous shaking. The cells were sedimented by centrifugation at 750*g* for 10 min, washed twice with 154 mM NaCl, 10 mM NaH_2PO_4, pH 7.3, and resuspended in the incubation medium (solution D; 145 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4, 1 mM $MgCl_2$, 10 mM NaMOPS, pH 7.4, 5.6 mM glucose, 0.5% BSA, 1 mM $CaCl_2$).

Duodenal cells were preequilibrated in the incubation medium for 10 min and then exposed for short intervals (30 s to 5 min) to 1α,25$(OH)_2D_3$ (0.01–10 nM), 25$(OH)D_3$ (1–100 nM), vitamin D_3 (100 nM), 25-hydroxy-16-ene-23-yne-D_3 (analog AT) (1 nM), 1,24-dihydroxy-22-ene-24-cyclopropyl D_3 (analog BT) (10 nM), 1α,25$(OH)_2$-lumisterol$_3$ (analog JN) (1 nM), 1α,25-dihydroxytachisterol$_3$ (analog JB) (1 nM), 1β,25$(OH)_2D_3$ (analog HL) (5 nM), or vehicle (ethanol) (<0.1%).

Cell viability was assessed by trypan blue exclusion. Morphological characterization of preparations was performed by phase-contrast microscopy.

Immunoprecipitation

After treatment, enterocytes were lysed (30 min at 4°C) in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EGTA, 25 mM NaF, 0.2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 2 µg/ml leupeptin, 2 µg/ml pepstatin, 2 µg/ml aprotinin, 0.25% sodium deoxycholate, and 1% NP40. Insoluble material was pelleted in a microcentrifuge at 14,000 rpm for 10 min. Proteins in the clear lysates were then quantitated according to Lowry [1951]. Aliquots (500–700 µg protein) were incubated overnight at 4°C with polyclonal antiphosphotyrosine or antiactive MAP kinase (Promega), which is reactive against both p42 and p44 isoforms of MAP kinase, followed by precipitation of the complexes with protein A conjugated with Sepharose. The immune complexes were washed five times with cold immunoprecipitation buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 0.2 mM sodium orthovanadate, 1% Triton X-100, and 1% NP40).

SDS-PAGE and Immunoblotting

Immunoprecipitated proteins (or lysate proteins) dissolved in Laemmli sample buffer were separated on SDS-polyacrylamide (7%) gels and electrotransferred to polyvinylidene difluoride (PVDF). Membranes were blocked for 1 h at room temperature in TBST (50 mM Tris-HCl, pH 7.4, 200 mM NaCl, 1% Tween-20) containing 1% dry milk. For the detection of tyrosine-phosphorylated proteins, membranes were subjected to immunoblotting using a rabbit antiphosphotyrosine antibody. Next, the membrane was washed three times in TBST, incubated with a 1:10,000 dilution of peroxidase-conjugated polyclonal antirabbit secondary antibody for 1 h at room temperature, and washed three additional times with TBST. The membrane was then visualized using an enhanced chemiluminescent technique (ECL) (Amersham Corp.) according to the manufacturer's intructions.

To strip the membrane for reprobing with antiactive MAP kinase, we washed the membrane 10 min in TBST and then incubated it in stripping buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, and 50 mM mercapthoethanol) for 30 min at 50°C. The membrane was again blocked and blotted as described above, except that the primary antibody used was a 1:1,000 dilution of antiactive MAP kinase.

Measurement of MAP Kinase Activity

Enterocytes were exposed either to 1α,25-$(OH)_2D_3$ (0.01–10 nM) for 1 min, 1α,25$(OH)_2D_3$ (1 nM) for 30 s to 5 min, or vehicle ethanol at 37°C. In some experiments, cells were pretreated with genistein (100 mM × 10 min). Lysates were prepared as described above. MAP kinase (p42 and p44) was immunoprecipitated from cell lysates as described above. After three washes in immunoprecipitation buffer and two washes in 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 phenylmethylsulfonyl fluoride, 20 µg/ml leupeptin, 20 µg/ml aprotinin, and 20 µg/ml pepstatin), 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, we separated the phosphorylated protein product from free [γ^{32}P]-ATP on ion-exchange phosphocellulose filters (P-81; Whatman, Clifton, NJ). Papers were immersed immediately in ice-cold 75 mM H_3PO_4, washed (1 × 5 min, 3 × 20 min), and counted in a scintillation counter.

RESULTS

In this work we have explored the complex signal transduction events in intestinal cells which may precede or contribute to the biological effects of the steroid hormone $1\alpha,25(OH)_2D_3$ which are mediated by its nuclear receptor, the VDR_{nuc}. To determine whether tyrosine phosphorylation in chick enterocytes could be modulated by the secosteroid hormone, cells were briefly stimulated with 1 nM $1\alpha,25(OH)_2D_3$ or vehicle (ethanol). Immunoprecipitation and Western blot analysis of enterocyte lysates with a polyclonal antiserum reactive with phosphotyrosine residues reveals that $1\alpha,25(OH)_2D_3$ causes a rapid increase (within 30 s) in tyrosine phosphorylation of several cellular proteins, including prominent bands at 40–44, 55–60, and 105–120 Kda (Fig. 1A). Because MAP kinase or extracellular signal-regulated kinase (ERK) consists of 42 Kda and 44 Kda isoforms and requires tyrosine phosphorylation for activation [Anderson et al., 1990], we examined whether the MAP kinase was phosphorylated after treatment with $1\alpha,25(OH)_2D_3$. Accordingly, the membrane was stripped and reprobed with antiactiveMAP kinase antibody which is reactive specifically against the 42 and 44 Kda species. Figure 1B illustrates that MAP kinase comigrated with the tyrosine-phosphorylated bands at an estimated molecular mass of 42 and 44 Kda. The response to physiological concentrations of the hormone is very rapid, with maximal levels of phosphorylation of MAP kinase observed at 1 min (three- to fourfold increase as shown by densitometric analysis of the data) (see Fig. 1B). Based on these results, subsequent MAP kinase phosphorylation studies were carried out using a 1 min time point.

To evaluate the dose dependence of $1\alpha,25(OH)_2D_3$ effects on MAP kinase tyrosine phosphorylation, proteins in cell lysates were separated by SDS-PAGE and immunoblotted with antiactive-MAP kinase antibody. Figure 2A shows hormone-induced MAP kinase tyrosine phosphorylation at 0.01 nM (onefold), with a maximal stimulation being achieved at 1 nM (fivefold). The effect of $1\alpha,25(OH)_2D_3$ was specific, since its metabolic precursors $25(OH)D_3$ and vitamin D_3 did not increase MAP kinase p42–44 tyrosine phosphorylation (Fig. 2B). A lower molecular mass protein (38 Kda) that appeared to be specific was detected by antiactive MAP kinase antibody in $25(OH)D_3$-treated cells and may represent a proteolytic product derived from the intact MAP kinase protein or the phosphorylation of a tyrosine residue of a MAP kinase isoform of 38 Kda. The extracellular regulated MAP kinase subfamily, besides the best known p42 and p44 (ERK1 and ERK2) signal-regulated species, includes, among others, p38 and p57 MAP kinases [Lee et al., 1993; Rouse et al., 1997]. These isoforms have been detected in mammals, although it is not known if they are present in avian species.

Several MAP kinases capable of phosphorylating myelin basic protein have been identified. These kinases are members of the extracellular signal-regulated kinase or ERK family [Boulton et al., 1991; Robbins et al., 1992]. To investigate whether $1\alpha,25(OH)_2D_3$ stimulates MAP kinase catalytic activity, we exposed enterocytes to the hormone followed by immunoprecipitation of the MAP kinase p42–44 species. Then, using the previously mentioned MAP kinase specific antibody, we assayed cell lysates for kinase catalytic activity in the presence of myelin basic protein as substrate (Fig. 3). $1\alpha,25(OH)_2D_3$ rapidly stimulated MAP kinase catalytic activity with kinetics comparable to the time course of appearance of the phosphorylated MAP kinase assayed via immunoblotting (Fig. 1B). Maximal activation was achieved after 1 min of hormone exposure (Fig. 3A). Figure 3B shows the dose dependence of MAP kinase activation by $1\alpha,25(OH)_2D_3$ with maximal stimulation obtained at 1 nM. Activation of MAP kinase was clearly dependent on the tyrosine kinase activity, as the tyrosine kinase inhibitor genistein suppressed MAP kinase activation in response to the hormone (Fig. 3C).

The best characterized signal transduction pathway for $1\alpha,25(OH)_2D_3$ in intestinal cells involves the stimulation of PLC and phosphoinositide hydrolysis to produce the dual second messengers, inositoltrisphosphate and diacylglycerol [Wali et al., 1990, 1992; de Boland et al., 1996], which is accompanied by a transient activation of PKC [Wali et al., 1990; Marinissen et al., 1994]. To investigate the possible role of PKC as an upstream mediator of the $1\alpha,25(OH)_2D_3$ signal, we exposed enterocytes to the phorbol ester PMA (100 nM) for 1 min. Parallel cells were pretreated (10 min) with the PKC inhibitor staurosporin (30 nM), which interferes with ATP binding to the catalytic domain of PKC [Hidaki and Hagiwara, 1987], followed by 1 min exposure to $1\alpha,25(OH)_2D_3$ (1

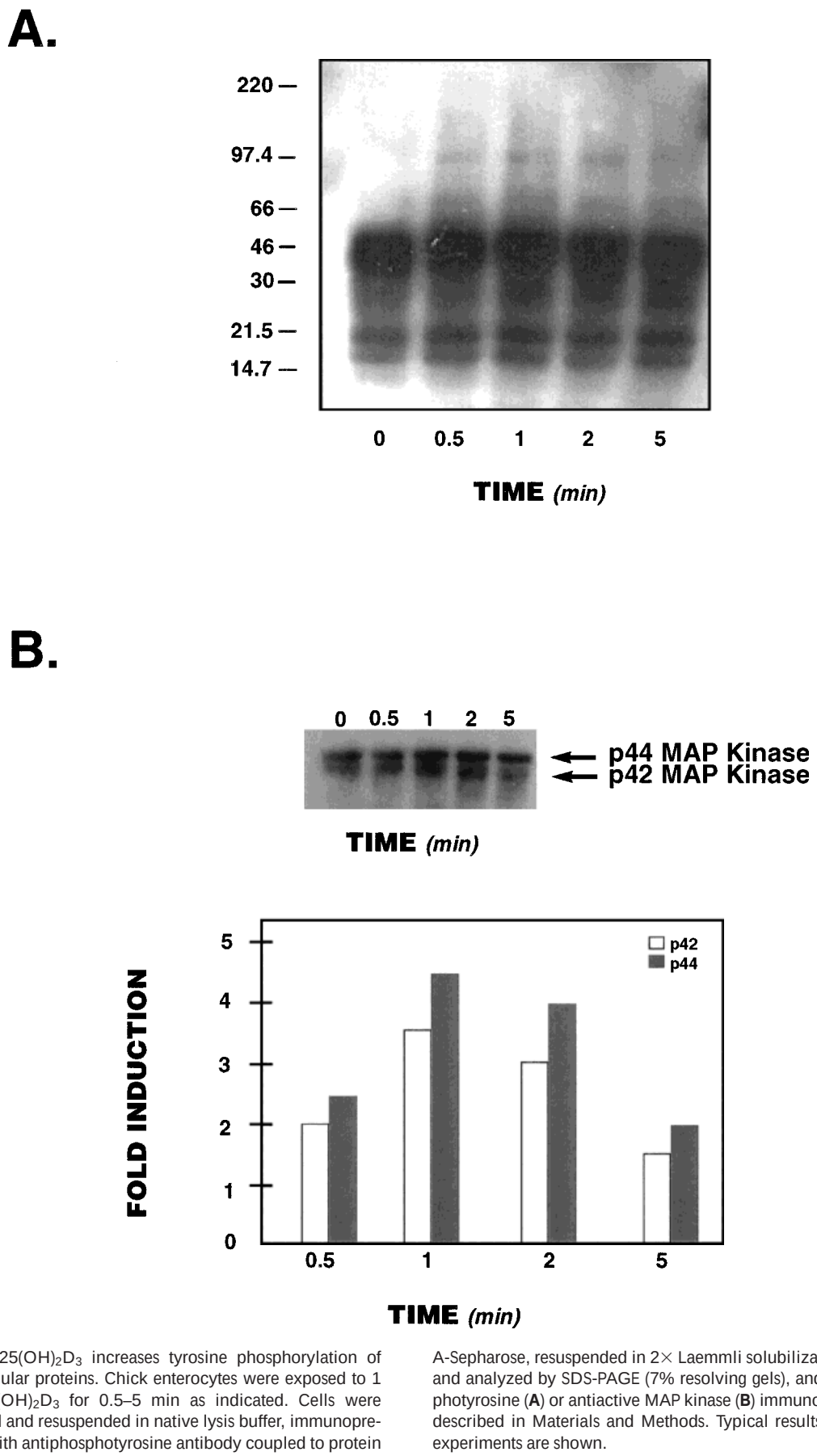


Fig. 1. $1,25(OH)_2D_3$ increases tyrosine phosphorylation of several cellular proteins. Chick enterocytes were exposed to 1 nM $1\alpha,25(OH)_2D_3$ for 0.5–5 min as indicated. Cells were sedimented and resuspended in native lysis buffer, immunoprecipitated with antiphosphotyrosine antibody coupled to protein A-Sepharose, resuspended in 2× Laemmli solubilization buffer, and analyzed by SDS-PAGE (7% resolving gels), and antiphosphotyrosine (**A**) or antiactive MAP kinase (**B**) immunoblotting as described in Materials and Methods. Typical results from two experiments are shown.

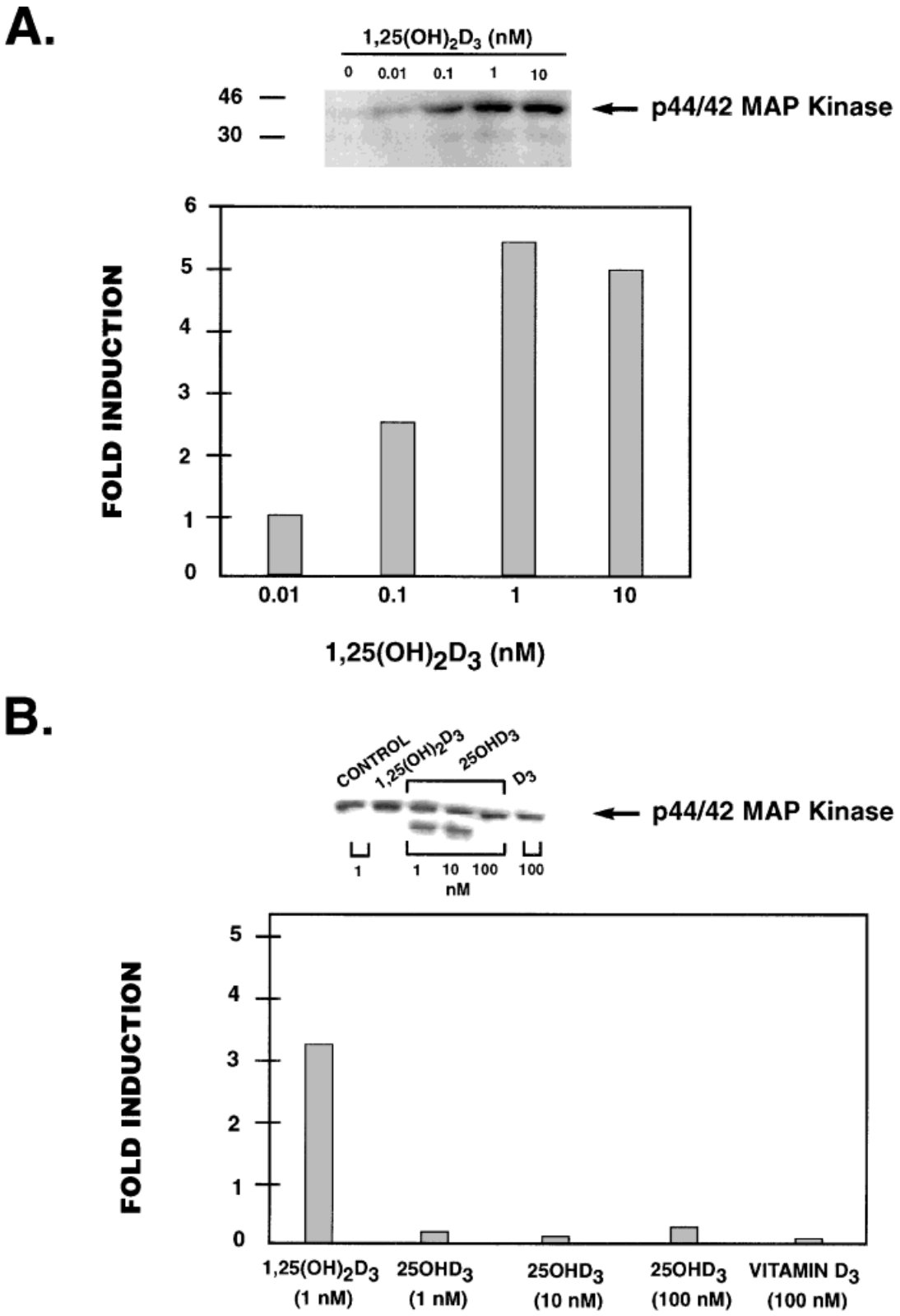


Fig. 2. 1α,25(OH)$_2$D$_3$ activation of enterocytes MAP kinase is dose-dependent and specific**.** Chick enterocytes were lysed following 1 min treatment with (**A**) ethanol vehicle, or 1,25(OH)$_2$D$_3$ (0.01–10 nM) or (**B**) ethanol vehicle, 1α,25(OH)$_2$D$_3$ (1 nM), 25(OH)D$_3$ (1–100 nM), or vitamin D$_3$ (100 nM). After lysing, comparable aliquots of cell proteins were mixed with 2× Laemmli solubilization buffer, heated (95°C × 5 min), and separated by SDS-PAGE (7% resolving gels). Each lane was then subjected to Western blotting with antiactive MAP kinase as described in Materials and Methods. Typical results from three experiments are presented.

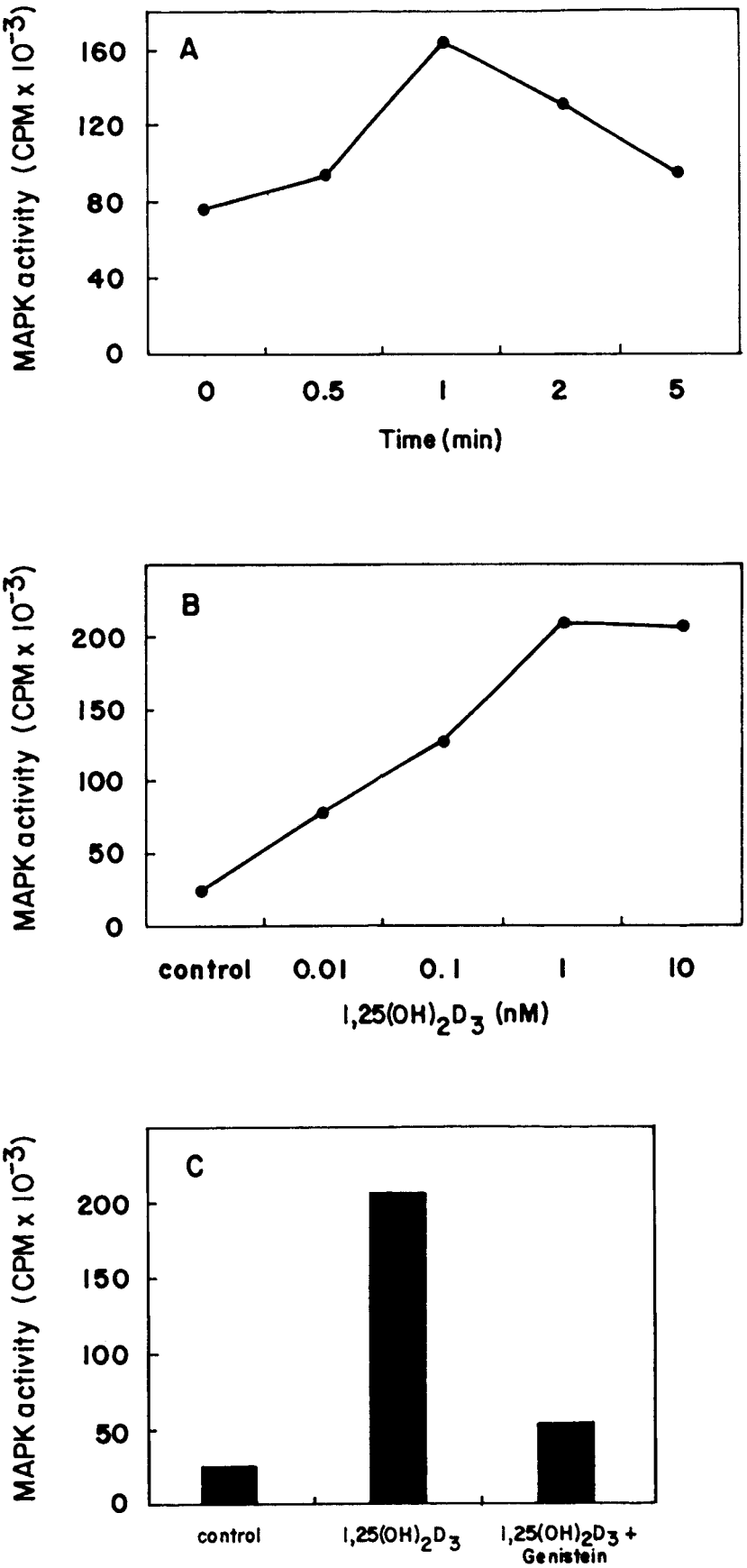


Fig. 3. Activation of MAP kinase by $1\alpha,25(OH)_2D_3$. Chick enterocytes were lysed following treatments with (**A**) $1\alpha,25(OH)_2D_3$ (1 nM) for 0.5–5 min as indicated, (**B**) $1\alpha,25(OH)_2D_3$ (0.01–10 nM) for 1 min, or (**C**) $1\alpha,25(OH)_2D_3$ (1 nM) for 1 min in the presence or absence of genistein (100 uM). Lysates were immunoprecipitated with antiactive MAP kinase antibody, and the catalytic kinase activity of the immunoprecipitate was measured using myelin basic protein as a substrate as described in Materials and Methods. Typical results from duplicate experiments are presented.

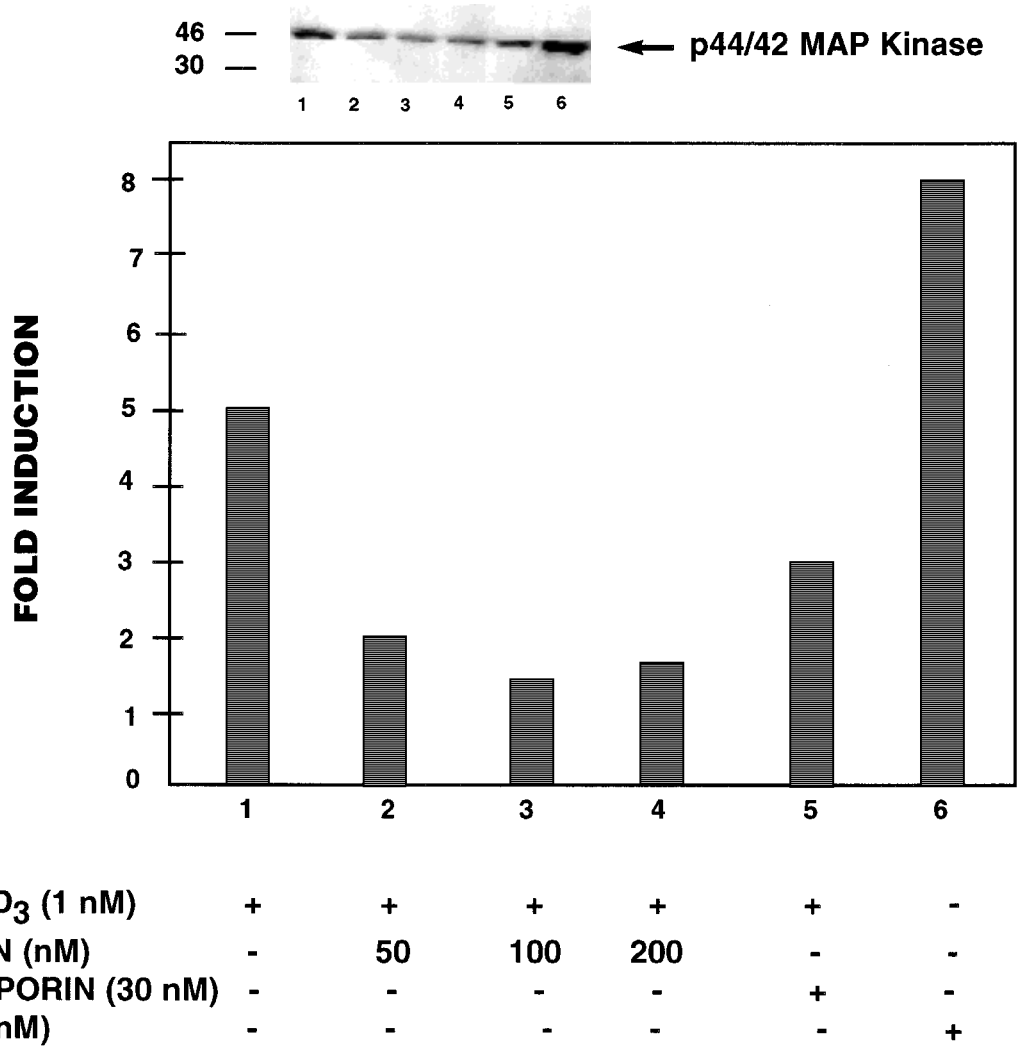


Fig. 4. 1α,25(OH)$_2$D$_3$-induced MAP kinase tyrosine phosphorylation is inhibited by the tyrosine kinases inhibitor genistein and the PKC inhibitor staurosporin. Chick enterocytes were lysed following 1 min treatment with 1α,25(OH)$_2$D$_3$ (1 nM) in the presence or absence of genistein (50–200 mM) or staurosporin (30 nM) or with PMA (100 nM) followed by SDS-PAGE and antiactive MAP kinase immunoblotting as described in Materials and Methods.

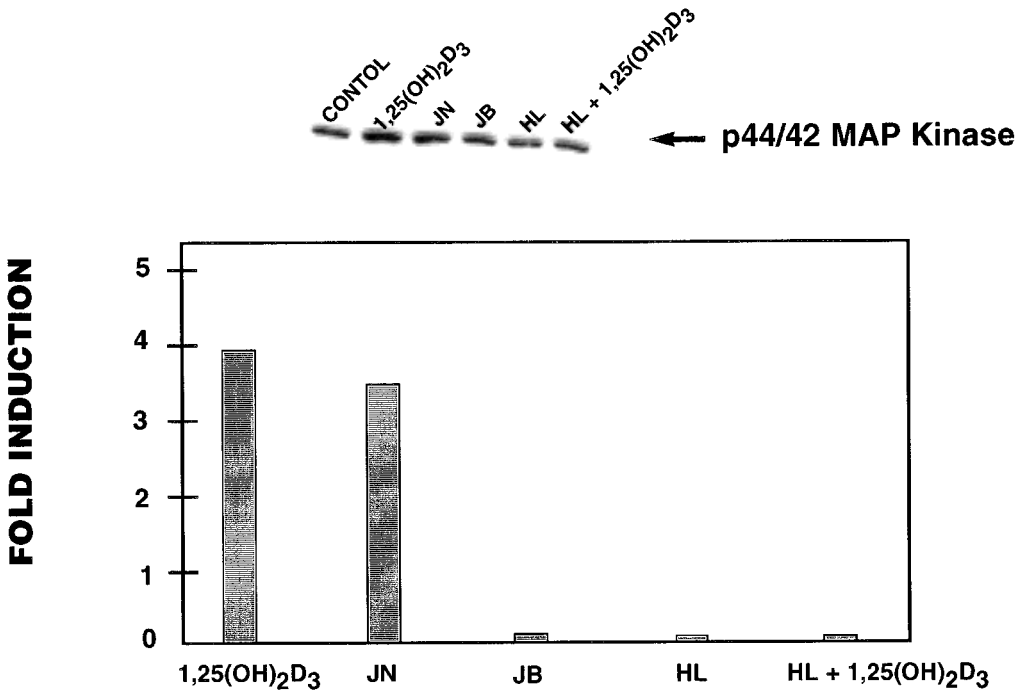
nM). As shown in Figure 4, acute PMA treatment results in a seven- to eightfold stimulation of MAP kinase tyrosine phosphorylation, while staurosporine partly abolished (≈30%) the hormone stimulation of the MAP kinase tyrosine phosphorylation.

In this study, we have evaluated the comparative activation of MAP kinase by a number of analogs of 1α,25(OH)$_2$D$_3$ which displayed complete flexibility around the 6,7 carbon-carbon bond (6F) or which were locked in either the 6-s-*cis* (6C) or 6-s-*trans* (6T) shape. The structures of these analogs are shown in Figure 5B. As shown in Figure 5A, analogs AT (25-hydroxy-16-ene-23-yne-D$_3$) (6F), BT (1α,24(OH)$_2$-22-ene-24-cyclopropyl-D3) (6F), and analog JN (1α,25(OH)$_2$-lumisterol$_3$) (6C) all stimulated while analog JB (1α,25-dihydroxytachisterol$_3$) (6T) did not stimulate MAP kinase tyrosine phosphorylation. In addition, analog HL (1β,25-(OH)$_2$D$_3$), a known antagonist of 1α,25(OH)$_2$D$_3$-mediated rapid responses [Norman et al., 1993; Zanello and Norman, 1997], blocked the hormone effects on MAP kinase.

DISCUSSION

In this work, we have explored the complex signal transduction events in intestinal cells which may precede or contribute to the biological effects of the steroid hormone 1α,25(OH)$_2$D$_3$ which are mediated by its nuclear receptor, the

A.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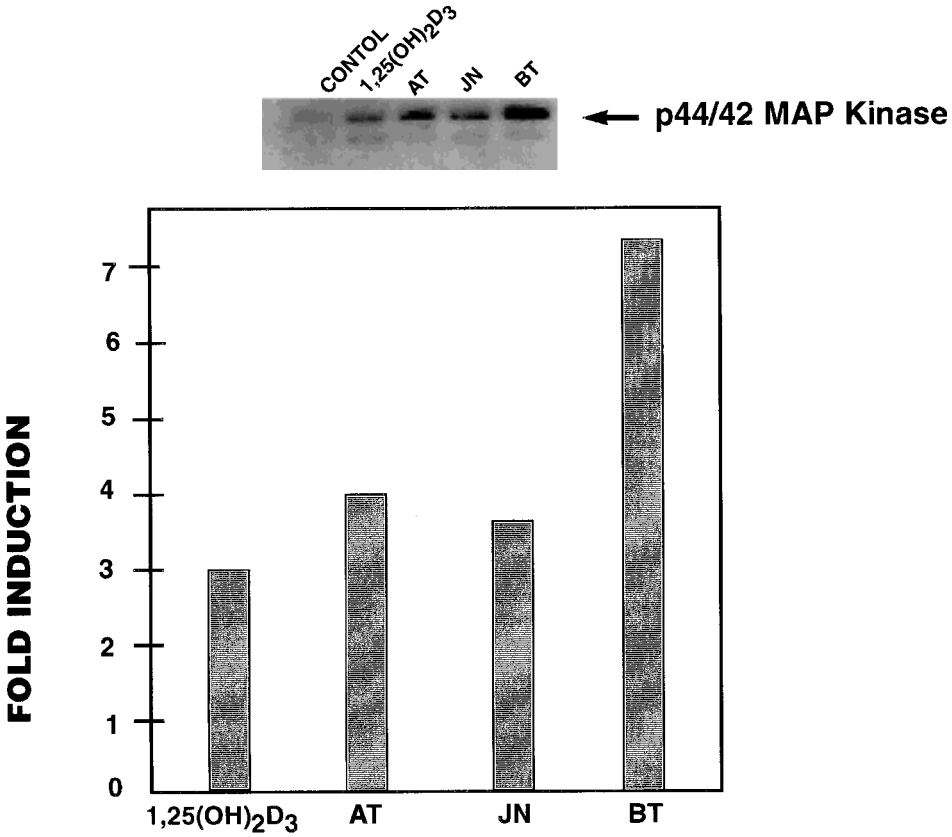
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Figure 5.

B.

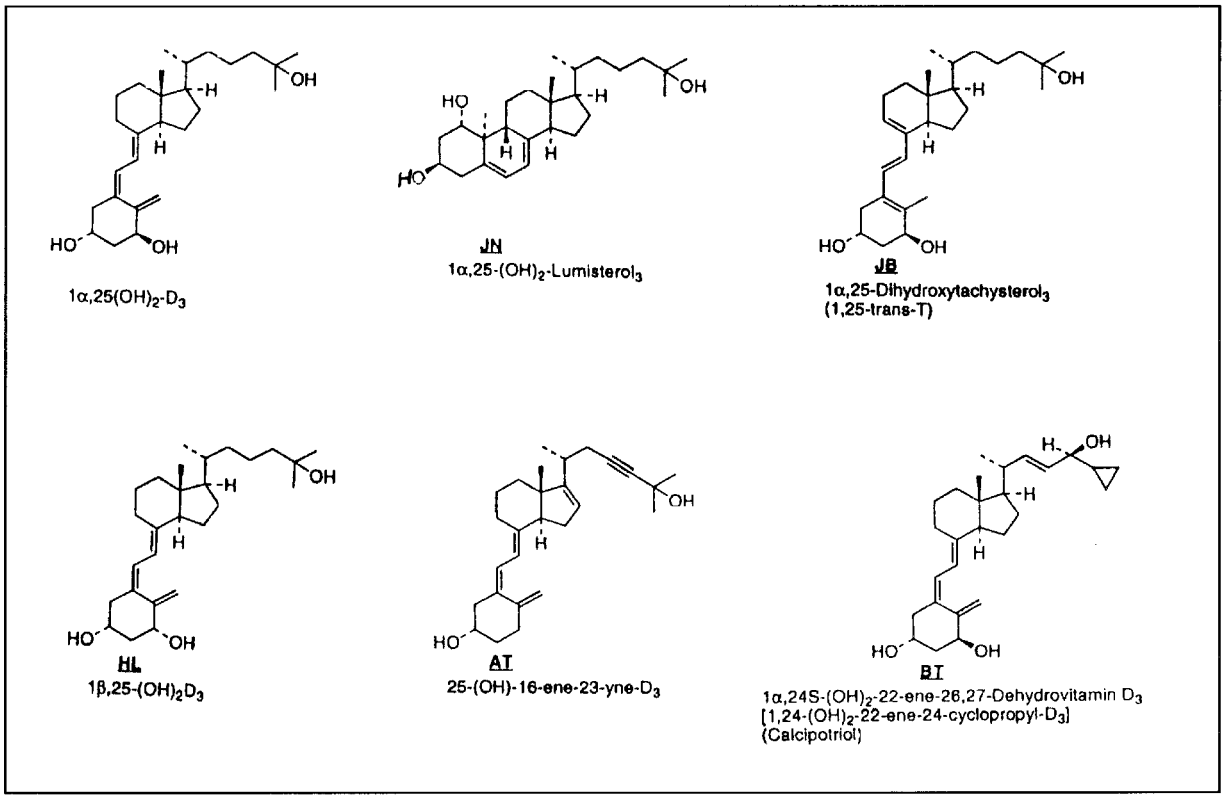

Fig. 5. Effects of 1α,25(OH)2D3 analogs on MAP kinase tyrosine phosphorylation**. A:** Chick enterocytes were lysed following 1 min separate exposure to 1α,25(OH)$_2$D$_3$, JN, JB, or AT (1 nM), BT (10 nM), and analog HL (5 nM) alone or HL (5 nM) + 1α,25(OH)$_2$D$_3$ (1 nM); this was followed by SDS-PAGE and antiactive MAP kinase immunoblotting as described in Materials and Methods. **B:** Structures of 1α,25(OH)$_2$D$_3$ and analogs utilized for the results presented in panel B. 1α,25(OH)$_2$D$_3$ is a conformationally flexible molecule, particularly because it is a seco steroid (see footnote 1) as a consequence of breaking the 9,10 carbon bond. All vitamin D seco steroids undergo a facile and rapid 360° rotation about the 6,7 carbon single bond; this generates a family of conformations extending, in the limit, from the steroid-like (6-s-*cis*) to the extended (6-s-*trans*) conformation. The analogs HL, BT, and AT all display conformational flexibility comparable to 1α,25(OH)$_2$D$_3$. In contrast, analog JN (1α,25(OH)$_2$-lumisterol$_3$), which is not a seco steroid, is permanently locked in the 6-s-*cis* conformation. Analog JB is permanently locked in the 6-s-*trans* shape because of the presence of a 6,7 carbon double bond. Analog HL is epimeric at carbon 1 with 1α,25(OH)$_2$D$_3$ (i.e., the orientation of the hydroxyl group on carbon 1 of HL is 1β rather than 1α).

VDR$_{nuc}$.Our data demonstrate, for the first time, that 1α,25(OH)$_2$D$_3$ stimulates MAP kinase activity in intestinal cells, which are classical target cells for the vitamin D hormone. The stimulation of MAP kinase by 1α,25(OH)$_2$D$_3$ was both dose- and time-dependent (Figs. 2, 3). Neither vitamin D or 25(OH)D$_3$ was effective at MAP kinase activation, suggesting that the effect may be limited to 1α,25(OH)$_2$D$_3$ and related analogs.

There are three unique structural features for the steroid hormone 1α,25(OH)$_2$D$_3$ which differentiate it from the rigid structures of the classical steroid hormones (e.g., estradiol, progesterone, cortisol, etc.). These include 1) the flexible 8-carbon side chain;, 2) the seco B ring which permits rotation around the 6,7 single carbon-carbon bond so as to generate a continuum of shapes extending from the limits of 6-s-*cis* to the 6-s-*trans* shapes (see Fig. 5A), and 3) the A ring which undergoes chair-chair conformational interconversion characteristic of cyclohexane rings. The conformational mobility of 1α,25(OH)$_2$D$_3$ allows generation of a wide array of conformers or shapes which are available for binding to potential ligand binding domains [Norman et al., 1996]. A key issue is whether both the genomic and rapid responses initiated by the ligand 1α,25(OH)$_2$D$_3$ are mediated by a ligand with the same conformation or

shape. This laboratory has previously suggested that the rapid responses were preferentially stimulated by ligands which are in the 6-s-*cis* shape [Norman et al., 1996].

Thus, in the present study (Fig. 5), only analogs locked in the 6-s-*cis* conformation (JN; 6C shape) or conformationally flexible analogs (AT, BT; 6F shape) but not analogs locked in the 6-s-*trans* shape (JB) were effective at MAP kinase activation. Rapid response effects of $1\alpha,25(OH)_2D_3$ that are mimicked by analogs which are 6-s-*cis*-locked and which have only 1–2% of the activity of $1\alpha,25(OH)_2D_3$ in binding in vitro to the nuclear receptor for $1\alpha,25(OH)_2D_3$ (VDR_{nuc}) or in regulating in vivo gene transcription have been recently reported [Norman et al., 1997]. Analog AT, which has conformational flexibility equivalent to $1\alpha,25(OH)_2D_3$, and analog JN, which is permanently locked in a 6-s-*cis* shape, have been shown to be full agonists of rapid responses [Norman et al., 1997]. We conclude that $1\alpha,25(OH)_2D_3$ and analogs that can achieve the 6-s-*cis* shape (both 6F and the permanently locked 6C) can increase tyrosine phosphorylation and activation of MAP kinase in chick enterocytes. In addition, the ability of the $1\beta,25(OH)_2D_3$ analog (HL) to block the rapid stimulation of MAP kinase by its epimer $1\alpha,25(OH)_2D_3$ is consistent with earlier reports describing its inhibitory actions on rapid responses of transcaltachia [Norman et al., 1993; Zanello and Norman, 1997].

Stimulation of MAP kinase activity has been also observed for $1\alpha,25(OH)_2D_3$ in nonclassical target cells such as keratinocytes [Gniadecki, 1996] and hepatic Ito cells [Beno et al., 1995]. As shown in Figure 4, genistein, in a dose-dependent manner, inhibited the ability of $1\alpha,25(OH)_2D_3$ to stimulate the phosphorylation of MAP kinase tyrosine; this is analagous to the genistein inhibition of the catalytic activity of the MAP kinase shown in Figure 3.

MAP kinases are a family of serine/threonine kinases that are regulated by threonine and tyrosine phosphorylation. MAP kinase (ERK1/ERK2) is phosphorylated by MAP kinase MEK, which in turn is activated upstream by Raf kinase [Egan et al., 1993]. Recent studies have demonstrated that $1,25(OH)_2D_3$ can activate Raf kinase in hepatic Ito cells [Lissoos et al., 1993]. The MAP kinase pathway is usually initiated by the phosphorylation in tyrosine of Shc proteins and their association with Grb2 and mSos, leading to the activation of Ras [Pelicci et al., 1992; Egan et al., 1993; Taniguchi, 1995]. This sequence of events has also been demonstrated in keratinocytes exposed to $1\alpha,25(OH)_2D_3$ [Assefa et al., 1997; Goldman et al., 1996].

MAP kinase may have roles in regulation of protein phosphorylation in the nucleus as well as in the cytoplasm [Pelech and Sanghera, 1992; Roberts, 1992]. Upon activation, MAP kinases can translocate to the nucleus, where they phosphorylate and activate nuclear transcription factors involved in DNA synthesis and cell division [Blenis, 1993]. In keratinocytes, $1\alpha,25(OH)_2D_3$ stimulates DNA synthesis via sequential activation of Raf and MAP kinase [Gniadecki, 1996]. The hormone has been shown to induce proto-oncogene expression in several cell types, and, in hepatic Ito cells, $1\alpha,25(OH)_2D_3$ requires MAP kinase activity to induce the nuclear proto-oncogene Erg expression [Beno et al., 1995]. As yet, the consequences of activation of the MAP kinase cascade in chick enterocyte are unclear. By analogy with other cell types, MAP kinase may be involved in the control of gene expression and cell growth. In this regard, it is interesting that estrogen can also rapidly activate MAP kinase in ROS17/2.8 osteoblast cells [Endoh et al., 1997]; this suggests that rapid activation of MAP kinase by steroid hormones may be a general feature of their actions.

The inhibition by staurosporine of PMA activation of MAP kinase (Fig. 4) suggests that PKC may be necessary but not sufficient for MAP kinase response to $1\alpha,25(OH)_2D_3$. Stimulation of MAP kinase and, further upstream, of Raf kinase by $1\alpha,25(OH)_2D_3$ via a PKC-dependent pathway have been recently reported in other cell types [Beno et al., 1995; Lissoos et al., 1993]. The mechanism by which PKC activates the MAP kinase cascade is unclear, although it has been suggested that PKC directly phosphorylates and activates Raf in a p21 ras-independent manner [Kolch et al., 1993]. PKC is used by many receptor types to regulate the MAP kinase pathway, alone or with other mechanisms, and may act at several steps in the cascade [Cobb and Goldsmith, 1995]. The pathway by which $1\alpha,25(OH)_2D_3$ activates MAP kinase in chick enterocytes remains to be determined, but it may involve a PKC-dependent and PKC-independent mechanism.

The mechanism of signal transduction from the surface of the enterocyte to the nucleus

remains to be fully elucidated. However, this laboratory has reported the partial purification from chick enterocyte basal lateral membranes of a binding protein for $1\alpha,25(OH)_2D_3$ that is different in its properties from the VDR_{nuc} [Nemere et al., 1994]. Future experiments are needed to dissect $1\alpha,25(OH)_2D_3$ upstream activation of the MAP kinase cascade and to elucidate the link between MAP kinase activation and the final transcriptional events.

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