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Non-genomic stimulation of tyrosine phosphorylation cascades by $1,25(OH)_2D_3$ by VDR-dependent and -independent mechanisms in muscle cells

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Abstract

Studies with different cell types have shown that modulation of various of the fast as well as long-term responses to $1,25(OH)_2D_3$ depends on the activation of tyrosine kinase pathways. Recent investigations of our laboratory have demonstrated that $1,25(OH)_2D_3$ rapidly stimulates in muscle cells tyrosine phosphorylation of PLC-γ and the growth-related proteins MAPK and c-myc. We have now obtained evidence using antisense technology indicating that VDR-dependent activation of Src mediates the fast stimulation of tyrosine phosphorylation of c-myc elicited by the hormone. This non-genomic action of $1,25(OH)_2D_3$ requires tyrosine phosphorylation of the VDR. Immunoprecipitation under native conditions coupled to Western blot analysis revealed $1,25(OH)_2D_3$-dependent formation of complexes between Src and the VDR and c-myc. However, the activation of MAPK by the hormone was only partially mediated by the VDR and required in addition increased PKC and intracellular Ca^{2+}. Following its phosphorylation, MAPK translocates into the nucleus where it regulates c-myc transcription. Altogether these results indicate that tyrosine phosphorylation plays a role in the stimulation of muscle cell growth by $1,25(OH)_2D_3$. Data were also obtained involving tyrosine kinases and the VDR in hormone regulation of the Ca^{2+} messenger system by mediating the stimulation of store-operated calcium (SOC; TRP) channels. Congruent with this action, $1,25(OH)_2D_3$ induces a rapid translocation of the VDR to the plasma cell membrane which can be blocked by tyrosine kinase inhibitors. Of mechanistic relevance, an association between the VDR and TRP proteins with the participation of the scaffold protein INAD was shown.

Keywords: $1,25(OH)_2D_3$; Non-genomic; Tyrosine phosphorylation; VDR; TRP; Muscle

1. Introduction

As in other target cells, $1,25(OH)_2D_3$ elicits long-term and short-term responses in muscle which involve genomic and non-genomic mode of actions, respectively [1]. In the first, more classic mechanism, through nuclear VDR-mediated gene transcription the hormone stimulates muscle cell proliferation and differentiation expressed by increased myoblast DNA synthesis followed by the induction of muscle specific myosin and calcium binding proteins [1,2]. $1,25(OH)_2D_3$ also exerts fast non-genomic actions in muscle cells which involve G protein-coupled stimulation of adenylyl cyclase and phospholipases C, D and A_2, and activation of protein kinases A and C which, in turn regulate the activity of voltage-dependent Ca^{2+} channels (VDCC) [1,3–5]. The hormone also promotes Ca^{2+} mobilization from intracellular stores and modulates store-operated Ca^{2+} (SOC) channels as part of the $1,25(OH)_2D_3$-induced Ca^{2+} entry across the plasma membrane of skeletal muscle cells [6]. This established role of the hormone in the regulation of muscle growth and intracellular Ca^{2+} homeostasis underlies the atrophy and alterations in contractility observed in states of $1,25(OH)_2D_3$ deficit [7].

There is evidence indicating that activation of tyrosine phosphorylation pathways may participate in the responses induced by $1,25(OH)_2D_3$ through its non-genomic mechanism. Tyrosine phosphorylation is a crucial event in signal transduction linked to the mitogen-activated protein kinase (MAPK). Stimulation of the MAPK cascade may occur through activation of receptor tyrosine kinases or G protein-coupled receptors by stimulation of non-receptor Src kinases or by direct signaling to Raf via PKC [8].

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$1,25(OH)_2D_3$ rapidly promotes tyrosine phosphorylation of MAPK in promyelocytic NB4 leukemia cells [9] and enterocytes [10]. Similar observations made in muscle cells implicated the MAPK pathway in hormone control of myoblast proliferation [11]. Fast $1,25(OH)_2D_3$-dependent increases in Src kinase activity have been observed in colonocytes [12], keratinocytes [13] and also in muscle cells [14], implying that the hormone activates the MAPK cascade by stimulating Src. Other major tyrosine-phosphorylated targets of $1,25(OH)_2D_3$ in muscle cells are PLCγ, known to mediate intracellular Ca^{2+} mobilization and external Ca^{2+} influx, and, surprisingly, the growth-related protein c-myc, a novel finding for which there is no information on the signaling component(s) leading to phosphorylation of its tyrosine residues [11].

Upon phosphorylation by mitogens, MAPK is translocated from the cytoplasm into the nucleus which results in the activation or induction of transcription factors leading to the expression of genes involved in control of cellular growth [8]. Moreover, in connection with agonist regulation of intracellular Ca^{2+}, it has been shown that tyrosine kinases (TK) may also modulate Ca^{2+} entry through SOC channels [15].

The rapid nature and specificity by which $1,25(OH)_2D_3$ activates TK cascades suggest that interaction with a plasma membrane receptor is responsible for the initiation of its effects. There is no evidence on the presence of such novel $1,25(OH)_2D_3$ membrane receptor in muscle although its existence has been reported as mediator of other signal transduction pathways in different cell systems [16,17]. In the present work, we have addressed the possibility that the nuclear VDR itself mediates in muscle cells rapid hormone-induced events related to tyrosine phosphorylation. Within this context, we have examined the interaction of $1,25(OH)_2D_3$ with key signaling components involved in activation of the MAPK pathway (Src, and its relationship to c-myc; PKC; Ca^{2+}) and SOC channels (TRP; INAD).

2. Experimental

2.1. Reagents and supplies

$1,25(OH)_2D_3$ was provided by Hoffmann-La Roche Ltd., Basel, Switzerland. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum and Lipofectin were from Gibco BRL (Gaithersburg, MD). Rabbit polyclonal anti-phosphotyrosine and rat monoclonal anti-VDR (chick) antibodies were obtained from Upstate Biotechnology Inc. (Lake Placid, NY). Anti-active (phospho)MAP kinase (reactive against p42 and p44 isoforms) and anti-Src mouse monoclonal antibodies were from Promega (Madison, WI). Anti-c-myc antibody was purchased from Oncogene Research Products (Cambridge, MA, USA). *Caliphora vicinia* anti-TRP and anti-INAD antibodies were from Dr. C. Montell (Johns Hopkins University, Baltimore, MD). Secondary goat, horse radish peroxidase-conjugated anti-rat antibody was from Affinity Bioreagents (Golden, CO). Secondary goat, peroxidase-labeled, anti-rabbit and anti-mouse antibodies and the (ECL) kit for enhanced chemiluminescence were obtained from Amersham Corp. (Arlington Heights, IL). [γ^{32}P]-ATP (3000 Ci/mmol) was from New England Nuclear (Chicago, IL). Oligodeoxynucleotides were synthesized by the DNAgency (Malvern, PA). All other reagents and specific inhibitors, of analytical grade, were from Sigma Chemical Co. (St. Louis, MO).

2.2. Cell culture and subcellular fractionation

Undifferentiated, myogenic chick skeletal muscle cells (myoblasts) were isolated from the breast muscle of 13-day-old chick embryos (*Gallus gallus*) essentially as described before [5], and cultured in DMEM containing 10% fetal bovine serum at 37°C under a humidified atmosphere (air/5% CO_2) until confluence (4–6 days after plating) before use. By this time, myoblasts become differentiated into myotubes with both biochemical and morphologic characteristics of adult skeletal muscle fibers [2].

For subcellular fractionation, the cells were collected in cold buffer composed of 10 mM Tris-HCl pH 7.4, 0.3 M sucrose, 5 mM DTT, 1 mM PMSF, 1 μg/ml aprotinin and 1 μg/ml leupeptin (buffer A), followed by homogenization in a glass homogenizer and centrifugation at 500 $\times$ g for 10 min. The pellet represented the nuclear fraction. The supernatant was centrifuged at 100 000 $\times$ g during 30 min. The supernatant was saved (cytosol) and the pellet was suspended in 1.5 ml of 15% sucrose in buffer A and layered over a discontinuous sucrose density gradient formed by adding 1.5 ml of 45% sucrose and 2.0 ml of 30% sucrose. The samples were centrifuged at 76 000 $\times$ g for 3 h. The plasma membranes included in the interface between the 15% and 30% sucrose solutions were collected and sedimented at 100 000 $\times$ g. The sediment was resuspended in buffer A.

2.3. Cell transfection

As in previous studies [18], oligodeoxynucleotides (ODNs) were incubated with Lipofectin in DMEM for 15 min at room temperature. Plates of subconfluent cells were washed to remove serum before addition of ODN-Lipofectin mixtures and incubation was performed for 12 h at 37°C. The ODN solution was removed, DME was added and the plates were placed into a metabolic incubator for an additional period of 36 h. Control treatments included DMEM or Lipofectin (in DMEM) only. ODN sequences with phosphorothioate linkages throughout the entire molecule were employed. Antisense ODNs against Src and the VDR were designed according to the sequences of the corresponding mRNA from *Gallus gallus*, as follows. Anti-Src: 5′-CACCACCATGGGGAGCAGCA-3′ (against the 95–114 nucleotide sequence containing the AUG region); anti-VDR: a

pool of anti-VDR1, 5′-TPGTCCTTGGTGATTTTG-CAPG-3′ (against the poly A region), anti-VDR2, 5′-TPC-GATGACTTTCTGCTGCTPC-3′ and anti-VDR3, 5′-TPC-CTTCATCAT CCCAATGTPC-3′ (against internal nucleotide sequences). Antisense ODNs against TRPs were composed of a pool of anti-TRP1: 5′-AGCAGGVCCAG-GAARATGTRGA A-3′ (V = A, G or C; R = A or G) anti-TRP2: 5′-CCATTGAAAGGAATGGCAGT-3′, anti-TRP3: 5′-CTAAGGCTAGGGACGACCGT-3′ and anti-TRP4: 5′-TTTTGGACTAGG AACTAGAC-3′. Antisense TRPs were designed against fragments of 440 bp (anti-TRP1, -TRP2 and -TRP3) and 220 bp (anti-TRP4) amplified by PCR from highly conserved sequences of TRP proteins from different species (Santillan G, Vazquez G and Boland R, submitted). Immunoblotting of cell lysates with the corresponding antibodies showed that the antisense ODNs selectively suppressed their targeted proteins.

2.4. Immunochemical analysis

Immunoprecipitation assays under denaturing conditions were performed as described elsewhere [14], by incubating lysate samples (500–700 μg protein) from control (ethanol) and 1,25$(OH)_2D_3$-treated muscle cells, overnight at 4°C with either anti-Src, anti-phosphotyrosine, anti-VDR or anti-TRP antibodies, followed by precipitation of the immunocomplexes with protein A-Sepharose. The precipitated complexes were washed five times with cold 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 0.2 mM PMSF, 0.2 mM OV, 1% Triton X-100 and 1% NP40.

Co-immunoprecipitation assays were performed under native conditions in order to preserve protein–protein associations, and were conducted essentially as described [19, 20] with minor modifications specified in a recent report [14]. Briefly, immunoprecipitation of the supernatants was performed with the indicated antibodies as described above, except that precipitated immunocomplexes were washed five times with phosphate-buffered saline (PBS) solution.

Western blot analysis of immunoprecipitated proteins were performed under conditions which have been previously described in detail [11]. After incubating overnight with the indicated antibody, the membranes were incubated with the corresponding secondary antibody. Immunoreactive proteins were then visualized by ECL. Images were obtained with a imaging densitomer scanning at 600 dpi and printing at the same resolution.

2.5. Measurement of SOC influx

To evaluate SOC influx, spectrofluorimetric analysis of changes in intracellular Ca^{2+} ($[Ca^{2+}]_i$) was performed in Fura-2-loaded cells as reported [6]. 1,25$(OH)_2D_3$ was added in the absence of extracellular Ca^{2+} and in the presence of inhibitors of VDCC (5 μM nifedipine and 5 μM verapamil)

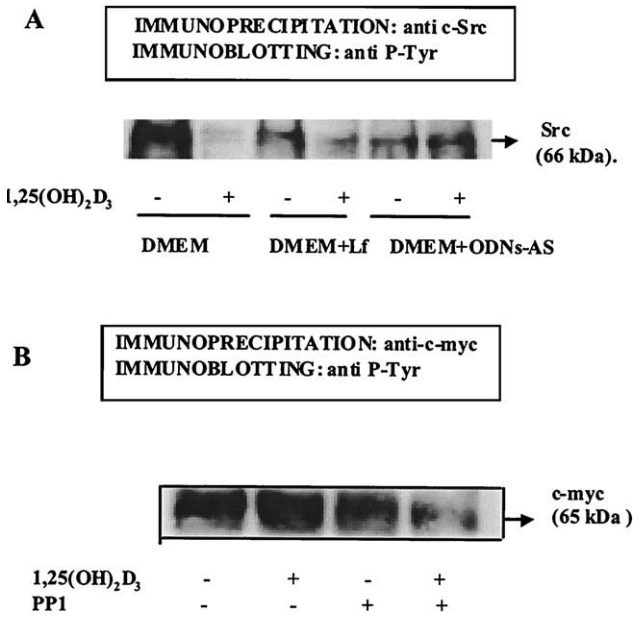

Fig. 1. A. Non-genomic stimulation of Src by 1,25$(OH)_2D_3$ is mediated by the VDR. Muscle cells were incubated for 48 h with DMEM or DMEM + Lipofectin (Lf) or antisense-VDR in DMEM + Lipofectin (DMEM + ODNs-AS). The cells were then treated for 1 min with 1 nM 1,25$(OH)_2D_3$, followed by immunoprecipitation with anti-cSrc and immunoblotting with anti-phosphotyrosine antibodies. B. 1,25$(OH)_2D_3$ stimulates tyrosine phosphorylation of c-myc through Src. Cells were exposed to 1,25$(OH)_2D_3$ ± 10 μM PP1, followed by immunoprecipitation with anti-c-myc and immunoblotting with anti-phosphotyrosine antibodies. Representative immunoblots from three independent experiments are shown.

to functionally isolate SOC channels. After liberation of Ca^{2+} from intracellular stores, revealed by a rapid and transient elevation in the fluorescence, Ca^{2+} (1.5 mM) was admitted to the medium (Ca^{2+} readdition protocol) which resulted in a fast and sustained increase in $[Ca^{2+}]_i$ due to Ca^{2+} entry through SOC channels [6]. SOC influx was also studied by Fura-2 fluorescence quenching by Mn^{2+} which selectively permeates this pathway. Once the store depletion-dependent transient Ca^{2+} rise induced by the hormone occurred, excitation was changed to 360 nm (isosbestic wavelength for Fura-2) followed by addition of Mn^{2+} (1 mM) to the medium, and Mn^{2+} influx followed as the rate of fluorescence quenching.

3. Results and discussion

In muscle cells 1,25$(OH)_2D_3$ stimulates within 1 min the activity of Src by decreasing its tyrosine phosphorylation (P-Tyr) state, the main mechanism of activation of the enzyme [14]. Fig. 1A shows that in cells which had been transfected with antisense sequences against the VDR (*see Experimental- 2.3*) the effects of 1,25$(OH)_2D_3$ on Src tyrosine dephosphorylation, measured by immunoprecipitation of Src coupled to immunoblotting with anti P-Tyr

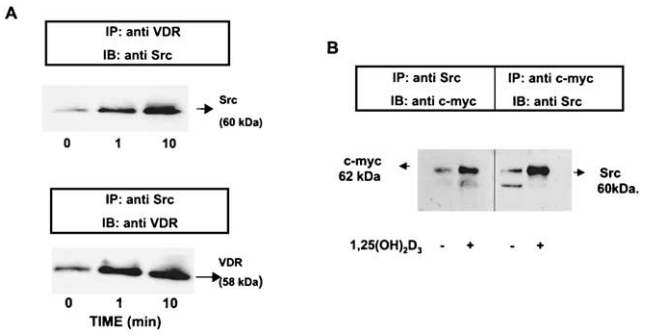

Fig. 2. Src associates with the VDR and c-myc. (A) Co-immunoprecipitation of Src and VDR. *Top*: Lysates from muscle cells exposed to $1,25(OH)_2D_3$ (1 nM) for 1 and 10 min, were immunoprecipitated with anti-VDR antibody under native conditions followed by immunoblot analysis with anti-Src antibody. *Bottom*: Immunoprecipitation was performed with anti-Src and immunoblotting with anti-VDR antibodies. (B) Co-immunoprecipitation of Src and c-myc. Cells were treated with 1 nM $1,25(OH)_2D_3$ for 1 min; the same procedure as in (A) was applied using anti-Src and anti-c-myc antibodies.

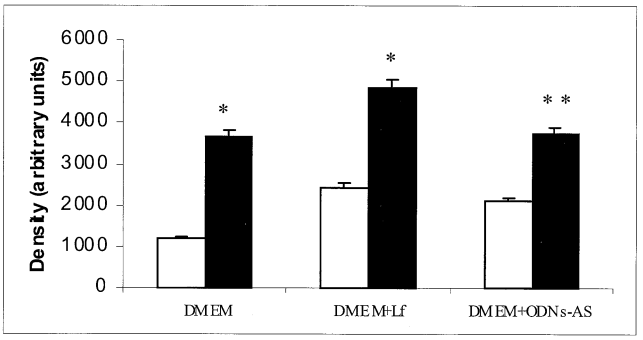

Fig. 3. The VDR partially mediates $1,25(OH)_2D_3$ activation of MAPK. Antisense ODNs against the VDR were used to block its expression as in Fig. 1. The cells were then treated with vehicle (□) or 1 nM $1,25(OH)_2D_3$ for 1 min (■), followed by immunoblot analysis with an anti-active MAPK antibody. Scanning densitometry of blots from three independent experiments is shown. * $P < 0.01$; ** $P < 0.05$, with respect to the corresponding control.

antibody, were significantly abolished. Clearly, the results of this experiment reveal that the VDR is involved in $1,25(OH)_2D_3$ stimulation of Src. We next pretreated muscle cells with the Src inhibitor PP1 (10 μM) followed by immunoprecipitation of c-myc and Western blotting with anti P-tyr antibody. Fig. 1B shows that PP1 suppressed hormone-induced tyrosine phosphorylation of c-myc [11]. Moreover, in cells transfected with the Src antisense-ODN the phosphorylation of c-myc induced by $1,25(OH)_2D_3$ was completely inhibited (not shown). Thus, one may conclude that Src activated through the VDR is involved in the non-genomic stimulation of c-myc by the hormone. Recent studies have demonstrated that the VDR rapidly undergoes tyrosine phosphorylation in response to $1,25(OH)_2D_3$ [14]. The fact that the VDR and c-myc behave as phosphotyrosine proteins opens the possibility that both macromolecules could be interacting with Src having a SH2 domain which recognizes phosphotyrosine residues. As shown in Fig. 2A and B, immunoprecipitation under native conditions coupled to Western blot analysis provided evidence on $1,25(OH)_2D_3$-dependent formation of complexes between Src and the VDR and c-myc.

Preincubation of muscle cells with compound PP1 or their transfection with an antisense ODN against Src mRNA significantly inhibited $1,25(OH)_2D_3$ activation of MAPK, involving Src as an upstream element leading to hormone signaling through this cascade (C. Buitrago, R. Boland, A.R. De Boland, submitted). However, the activation of MAPK by $1,25(OH)_2D_3$ was only partially suppressed (−35%) in cells in which expression of the VDR had been blocked with antisense ODNs against the VDR (Fig. 3). Therefore, experiments were performed to find out whether stimulation of MAPK by $1,25(OH)_2D_3$ could in addition be mediated by PKC signaling or through changes in intracellular Ca^{2+}. As shown in Fig. 4A, the hormone response was shown to depend on PKC stimulation since it was attenuated by the PKC inhibitors calphostin C (100 nM) and bisindolylmaleimide I (30 nM). Inhibition of PLC by neomycin also decreased hormone activation of MAPK whereas the Ca^{2+}-mobilizing agent thapsigargin and the Ca^{2+}-ionophore A23187 paralleled the phosphorylation of MAPK by $1,25(OH)_2D_3$, their effects being reduced by removal of external Ca^{2+} with EGTA (Fig. 4B).

In muscle cells stimulated with $1,25(OH)_2D_3$, markedly

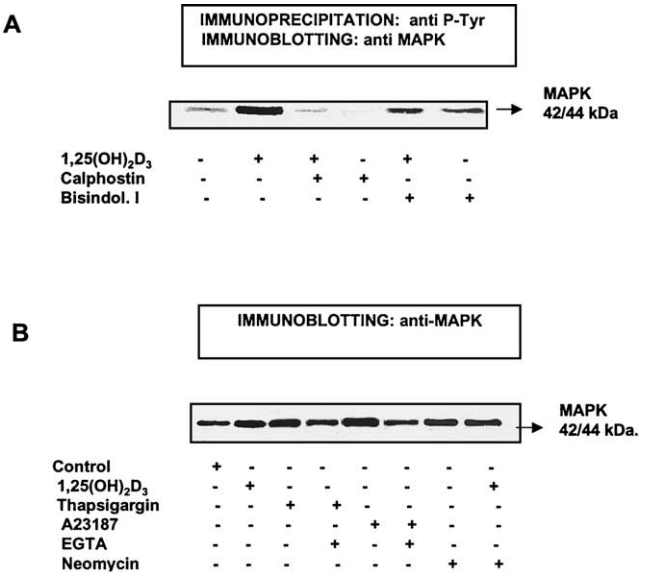

Fig. 4. PKC and Ca^{2+} also mediate $1,25(OH)_2D_3$ stimulation of MAPK. (A) Cells were incubated for 1 min with $1,25(OH)_2D_3$ (1 nM) in the absence and presence of calphostin C (100 nM) or bisindolylmaleimide I (30 nM). Lysates were immunoprecipitated with anti-phosphotyrosine followed by immunoblot analysis with anti-active MAPK antibodies. (B) The cells were treated with 1 nM $1,25(OH)_2D_3$, 1 μM thapsigargin or 1 μM ionophore A23187 with or without 0.5 mM EGTA or 0.5 mM neomycin for 1 min. Immunoblot analysis was performed employing an anti-active MAPK antibody. Representative immunoblots from three independent experiments are shown.

increased (98%) amounts of MAPK could be detected between 3 and 10 min in the nuclear fraction by immunoblotting with anti-active MAPK antibody. Parallelly, the expression of c-myc, measured by Western blot analysis, was augmented by 1,25$(OH)_2D_3$ almost 1-fold (+ 90%; $P < 0.01$) with respect to basal levels. When the cells were incubated with 1,25$(OH)_2D_3$ in the presence of the MAPK kinase (MEK) inhibitor PD98059 the hormone response was markedly reduced (+ 23% with respect to PD98059 alone; $P < 0.05$), thereby involving MAPK in 1,25$(OH)_2D_3$ regulation of c-myc expression.

The above results altogether indicate that stimulation of tyrosine phosphorylation cascades by 1,25$(OH)_2D_3$ partially mediated by the classic intracellular vitamin D receptor is involved in non-genomic regulation of muscle cell growth.

Evidence was also obtained showing that tyrosine kinases and the VDR are part of the non-genomic mechanism by which 1,25$(OH)_2D_3$ regulates the calcium messenger system in muscle cells modulating Ca^{2+} entry through store operated calcium (SOC) channels. SOC influx becomes activated as a consequence of a decrease and/or depletion of the Ca^{2+} content of the lumen of the endoplasmic reticulum [6 and references therein]. In various mammalian cell types, TRP proteins (homologous to the 'transient receptor potential' proteins of *D. melanogaster*) have been shown to function as channels mediating the SOC Ca^{2+} entry pathway. A plethora of messenger systems have been related to SOC influx regulation [19,20]. In Fura-2-loaded muscle cells by applying the spectrofluorimetric Ca^{2+} readdition protocol (*Experimental- section 2.5*), we generated evidence involving the participation of tyrosine kinase (TK)-mediated protein phosphorylation in this mechanism as we showed that the TK inhibitor genistein (50–100 μM) almost completely abolished 1,25$(OH)_2D_3$ stimulation of SOC influx (Fig. 5A). Similar results were obtained with herbimycin (10–50 μM), a highly specific blocker of tyrosine phosphorylation (not shown). In support of a role of the VDR in mediating hormone activation of Ca^{2+} entry through SOC channels, we observed that in muscle cells transfection with anti-VDR antisense ODN inhibited Fura-2 fluorescence quenching by Mn^{2+}, a selective tracer for SOC Ca^{2+} influx (Fig. 5B). Congruent with this action, 1,25$(OH)_2D_3$ induces a rapid translocation of the VDR from the nucleus to the plasma cell membrane which could be blocked by the TK inhibitor genistein whereas daidzein, an inactive analog, was without effects (Fig. 6). Moreover, of relevance for the mechanism of 1,25$(OH)_2D_3$ stimulation of SOC channels, a hormone-dependent association between the VDR and TRP proteins could be demonstrated by co-immunoprecipitation under native conditions (Fig. 7).

Finally, in both invertebrate photoreceptor cells as well as some vertebrate systems, TRP proteins have been shown to interact with PDZ domain-containing proteins with adaptor function, from which INAD constitutes the best characterized one. When muscle cells were permeabilized with saponin in the presence of a specific anti-INAD antibody,

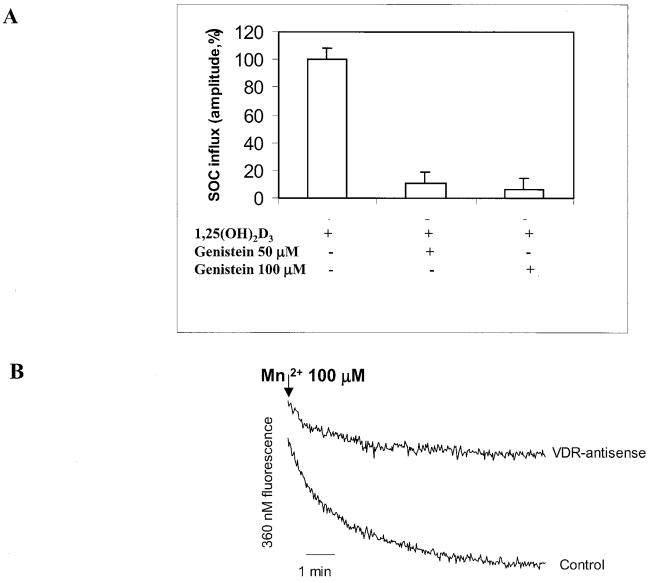

Fig. 5. Tyrosine phosphorylation and the VDR are required for non-genomic stimulation of SOC (TRP) influx by 1,25$(OH)_2D_3$. (A) 1,25$(OH)_2D_3$-induced SOC influx was measured by the Ca^{2+} readdition protocol (*section 2.5*). The tyrosine kinase inhibitor genistein was added after the initial hormone-dependent Ca^{2+} transient occurred. Averages of 5–8 recordings are given. (B) SOC influx was measured by Fura-2 fluorescence quenching by Mn^{2+} in control cells and cells transfected with antisense ODNs against the VDR as in Figs. 1 and 3.

the 1,25$(OH)_2D_3$-dependent SOC influx was almost totally suppressed, whereas permeabilization in the presence of normal rabbit IgG was without effect on the hormone stimulated SOC entry pathway (not shown). It is then possible that complexes formed by the VDR, TRP proteins and other signaling molecules, e.g. tyrosine kinases, by anchoring in INAD, mediate the stimulation of SOC influx by 1,25$(OH)_2D_3$.

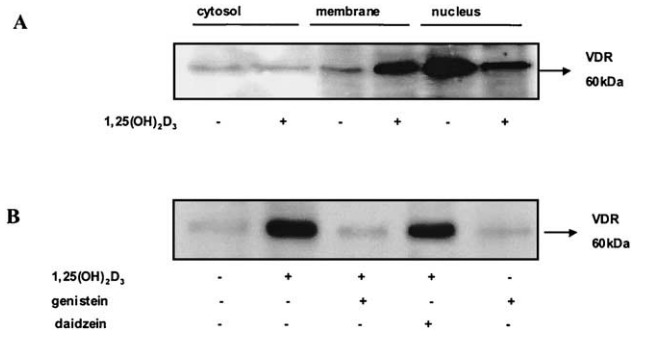

Fig. 6. 1,25$(OH)_2D_3$ translocates the VDR to the plasma membrane through a tyrosine-phosphorylation-dependent mechanism. A. Cytosol, plasma membranes and nucleus were isolated from control and 1,25$(OH)_2D_3$-treated muscle cells (1 nM, 1 min). B. Plasma membranes from control and treated cells in the absence or presence of genistein and its inactive analog daidzein. The VDR was detected by Western blot analysis. Representative immunoblots from three independent experiments are shown.

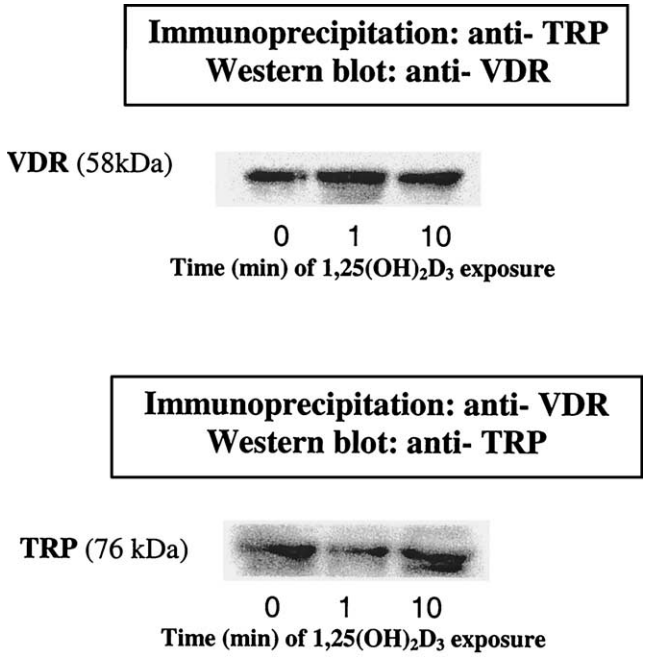

Fig. 7. $1,25(OH)_2D_3$-induced complex formation between the VDR and TRP proteins. *Top*: Lysates from muscle cells exposed to $1,25(OH)_2D_3$ (1 nM) for the indicated times were immunoprecipitated with anti-TRP antibody under native conditions followed by immunoblot analysis with anti-VDR antibody. *Bottom*: As above, but immunoprecipitation was performed with anti-VDR and immunoblotting with the anti-TRP antibodies.

Acknowledgments

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