Involvement of Tyrosine Kinase Activity in 1α,25(OH)₂-vitamin D₃ Signal Transduction in Skeletal Muscle Cells*

Received for publication, March 10, 2000, and in revised form, August 3, 2000
Published, JBC Papers in Press, August 29, 2000, DOI 10.1074/jbc.M00205200

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In cultured chick skeletal muscle cells loaded with Fura-2, the tyrosine kinase inhibitors herbimycin A and genistein abolished both the fast inositol 1,4,5-triphosphatase-dependent Ca²⁺ release from internal stores and extracellular Ca²⁺ influx induced by 1α,25(OH)₂-vitamin D₃ (1α,25(OH)₂D₃). Daidzein, an inactive analog of genistein, was without effects. Tyrosine phosphatase inhibition by orthovanadate increased cytosolic Ca²⁺. Anti-phosphotyrosine immunoblot analysis revealed that 1α,25(OH)₂D₃ rapidly (0.5–10 min) stimulates in a concentration-dependent fashion (0.1–10 μM) tyrosine phosphorylation of several myoblast proteins, among which the major targets of the hormone could be immunologically identified as phospholipase Cγ (127 kDa), which mediates intracellular store Ca²⁺ mobilization and external Ca²⁺ influx, and the growth-related proteins mitogen-activated protein (MAP) kinase (42/44 kDa) and c-myc (65 kDa). Genistein suppressed the increase in phosphorylation and concomitant elevation of MAPK activity elicited by the sterol. Both genistein and the MAPK kinase (MEK) inhibitor PD98059 abolished stimulation of DNA synthesis by 1α,25(OH)₂D₃. The sterol-induced increase in tyrosine phosphorylation of c-myc, a finding not reported before for cell growth regulators, was totally suppressed by the specific Src inhibitor PP1. These results demonstrate that tyrosine phosphorylation is a previously unrecognized mechanism of 1α,25(OH)₂D₃ regulation of Ca²⁺ homeostasis in hormone target cells. In addition, the data involve tyrosine kinase cascades in the mitogenic effects of 1α,25(OH)₂D₃ on skeletal muscle cells.

1α,25-Dihydroxy-vitamin D₃ (1α,25(OH)₂D₃) in addition to its classical role in the regulation of extracellular calcium homeostasis, modulates cell proliferation and differentiation and the immune system (1–5). The hormone also regulates skeletal muscle functions. Muscle weakness and atrophy are observed in vitamin D deficiency states and impaired metabolism. This myopathy is reversed by administration of physiological amounts of 1α,25(OH)₂D₃ (Refs. 6–8; for a review on this topic, see Ref. 9). Studies with animal models and cultured muscle cells have shown that the hormone exerts direct effects on skeletal muscle Ca²⁺ metabolism, contractility, and growth (9–11). As in other target cells (12–15), 1α,25(OH)₂D₃ elicits responses in muscle both through nuclear receptor-mediated gene transcription and a fast mechanism independent of new RNA and protein synthesis (11, 16). The non-genomic actions of 1α,25(OH)₂D₃ in muscle cells involve G protein-coupled stimulation of adenyl cyclase and phospholipases C, D, and A₂ and activation of protein kinases A and C which, in turn regulate the activity of voltage-dependent Ca²⁺ channels (VDCC) (17–22). The hormone also promotes Ca²⁺ mobilization from intracellular stores and modulates store-operated Ca²⁺ (SOC) channels as part of the 1α,25(OH)₂D₃-induced Ca²⁺ entry across the plasma membrane of skeletal muscle cells (23, 24). The rapid nature and specificity by which 1α,25(OH)₂D₃ 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 1α,25(OH)₂D₃ in skeletal muscle (25) as well as for this and other steroid hormones in various cell types (reviewed in Refs. 26 and 27) has been described. In connection to the muscle growth-promoting activity of 1α,25(OH)₂D₃ various lines of evidence have shown that the hormone stimulates both the proliferation and differentiation of myoblasts into myotubes (28–30).

Tyrosine phosphorylation is a crucial event in signal transduction mechanisms linked to the mitogen-activated protein kinase (MAPK) cascade 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 (31, 32). There is also evidence indicating that tyrosine kinases may modulate Ca²⁺ entry both through the VDCC (33, 34) and SOC channel (35–37) pathways. Variations in cytosolic Ca²⁺ levels are also of importance in the control of the cell cycle (38). In line with the participation of this mechanism, we recently obtained preliminary evidence indicating that in skeletal muscle cells tyrosine kinase phosphorylation of cellular proteins seems to play a role in 1α,25(OH)₂D₃-dependent modulation of non-genomic responses, such as fast increases in cytosolic Ca²⁺ and MAPK stimulation (39). Accordingly, it has been recently reported that 1α,25(OH)₂D₃ rapidly stimulates MAP kinase phosphorylation in both promyelocytic NB4 leukemia cells (40) and enterocytes (41). On these bases, in the present study we examined the participation of tyrosine kinase(s) in the mechanism by which 1α,25(OH)₂D₃ regulates cytoplasmic Ca²⁺ and
exerts mitogenic effects in skeletal muscle cells and investigated hormone-dependent related changes in protein tyrosine phosphorylation.

**EXPERIMENTAL PROCEDURES**

**Chemicals—**1α,25(OH)2-vitamin D3 was kindly provided by Dr. Heinrich Bachmann (Hoffman-La Roche Ltd., Basel, Switzerland). Fura-2/pentaacetoxymethyl ester (Fura-2/AM), phorbol 4,12-dibutyrate (PDBu), and PPI were supplied by CALBIOCHEM-Novabiochem and Pfizer, respectively. Rabbit polyclonal anti-phospho-tyrosine antibody was obtained from Upstate Biotechnology Inc. (Lake Placid, NY). Anti-MAP kinase antibody and the anti-phospho-MAP kinase, an antibody to the phosphorylated form of MAP kinase, was purchased from Oncogene Research Products (Cambridge, MA). Anti-phospholipase C was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Secondary antibody goat anti-rabbit horseradish peroxidase-conjugated IgG and the Super Signal CL-HRP substrate for chemiluminescence were obtained from PerkinElmer Life Sciences. Protein A-Sepharose was purchased from Pierce. All antibodies were of analytical grade.

**Cell Culture—**Chick skeletal muscle cells were obtained from 13-day-old chick embryo breast muscles by stirring in Earle’s balanced salt solution containing 0.1% trypsin for 30 min as essentially described previously (42). The freed cells were collected by centrifugation, and the pellet was resuspended in DMEM supplemented with 10% fetal bovine serum and antibiotic-antimycotic solution. The suspension was dispersed by pipette, filtered through nylon mesh, and "preplated" on gelatin-coated Petri dishes to remove contaminating fibroblasts. The adherent cells were allowed to grow until confluence (4–6 days after plating) before use. Under these conditions, myoblasts proliferate within the first 48 h and at day 4 become differentiated into myotubes expressing both biochemical and morphological characteristics of adult skeletal muscle fibers (43).

**Thymidine Incorporation—**The rate of thymidine incorporation into DNA was determined by adding 2 μCi of [3H]thymidine (20 Ci/mmol)/ml Dulbecco’s modified Eagle’s medium to muscle cell monolayers cultured (44). Cell dye loading was achieved by incubating the muscle cells in buffer A containing 138 mM NaCl, 5 mM KCl, 1 mM MgCl2, 5 mM glucose, 10 mM Hepes, pH 7.4, 1.5 mM CaCl2 plus 0.1% bovine serum albumin, 4 μM penta-acetoxyethylmeter derivative (membrane-permeable) Fura-2/AM, and 0.012% pluronic acid in the dark for 40 min at room temperature in order to minimize dye compartmentalization. Unloaded dye was washed out, and cells were maintained in buffer B (buffer A without bovine serum albumin, Fura-2/AM, and pluronic acid) in the dark (room temperature) for at least 40 min before use to allow for complete intracellular dye deesterrification. Coverslips containing confluent cells were placed into quartz cuvettes of a thermostatically controlled (37°C) SLM Amino 5100 spectrophotometer (Spectronics Inc.) sample compartment under constant, controlled stirring. Fura-2 intracellular fluorescence intensity was monitored at an emission wavelength of 510 nm (8-nm bandwidth) by alternating (300 Hz) the excitation wavelength between 340 and 380 nm (4-nm bandwidth) with a dual excitation monochromator.

Signals from short and long wavelengths were compared in a ratio (r = R510/R380) thus making the measurement independent of variations in cellular dye loading, dye leakage, or photobleaching. Calibration of Fura-2 fluorescence signal to calculate [Ca2+]i values was performed for each coverslip essentially as described by us (23, 24). Maximal (Rm) and minimal (Rm) intracellular dye fluorescence signals were determined by adding 5 μM ionomycin plus 3 mM CaCl2 and 10 mM EGTA, pH 9.0, respectively. Under these conditions of measurement, the dissociation constant (Kd) for the Ca2+/Fura-2 complex was assumed to be 224 nm, and [Ca2+]i, according to the algorithm of Grynkiewicz et al. (44) derives from [Ca2+]i = Kd (R - Rm)/(Rmax - R) × β, where β is the ratio of Fura-2 fluorescence at the selected wavelengths, Rmax and Rm represents ratios from Ca2+-saturated and Ca2+-free intracellular dye, respectively, and β is the ratio between the specific fluorescence of the Ca2+-bound and Ca2+-bound forms of the dye at the longer wavelength (Sβ/β).

In some experiments, a Ca2+-free extracellular medium was used. In such situations, the absence of Ca2+ in the medium means free Ca2+ concentration near 1 mM, which is accomplished by preparing a nominally Ca2+-free buffer B (see composition above) plus 1 mM EGTA. Free Ca2+ levels were calculated by using the WinMaxc program, version 1.7 (45). All buffers and saline solutions used were prepared with deionized water.

**Immunoprecipitation—**After treatment, muscle cells were lysed (30 min at 4°C) in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 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% Nonidet P-40. Insoluble material was pelleted in a microcentrifuge at 12,000 × g for 10 min. The protein content of the clear lysates was determined according to Lowry et al. (46). Aliquots (500–700 μg of protein) were incubated overnight at 4°C with anti-phospho-tyrosine, anti-phospho-MAP kinase (p42 and p44 isoforms), anti-PLCγ, or anti-c-myc antibodies followed by precipitation of each complex with protein A conjugated with Sepharose 4B. After washing five times with cold immunoprecipitation buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 0.2 mM sodium orthovanadate, 1% Triton X-100, and 1% Nonidet P-40).

**SDS-PAGE and Immunoblotting—**Immunoprecipitated proteins (or lysate proteins) dissolved in Laemmli sample buffer were separated on SDS-polyacrylamide (7%) gels (47) and electrotransferred to polyvinylidene difluoride membranes. The 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 anti-phospho-tyrosine antibody. The next, the membranes were washed three times in TBST, incubated with a 1:10,000 dilution of anti-phospho-MAP kinase (p42 and p44 isoforms), anti-PLCγ, or anti-c-myc antibodies followed by precipitation of each complex with protein A conjugated with Sepharose 4B. After washing five times with cold immunoprecipitation buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 0.2 mM sodium orthovanadate, 1% Triton X-100, and 1% Nonidet P-40). To strip the membrane for reprobing with anti-phospho-MAP kinase, the membrane was washed 10 min in TBST and then incubated in stripping buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, and 50 mM mercaptoethanol) for 30 min at 50°C. The membrane was again blocked and blotted as described above, except that the primary antibody used was a 1:1000 dilution of anti-phospho-MAP kinase.

**Statistical Analysis—**Statistical significance of the data was evaluated using Student’s t test (48), and probability values below 0.05 (p < 0.05) were considered significant. Results are expressed as means ± S.D. from the indicated set of experiments.

**RESULTS**

Stimulation of chick embryonic skeletal muscle cells with 1α,25(OH)2-vitamin D3 triggers a rapid (30 s) and sustained increment in intracellular calcium concentration ([Ca2+]i) that persists
elevated as long as the cells are exposed to the hormone (Fig. 1). We have previously shown that the rapid initial \([Ca^{2+}]_i\) response to the sterol mainly results from inositol 1,4,5-trisphosphate-mediated mobilization of \(Ca^{2+}\) from a thapsigargin-sensitive store, whereas the plateau phase is entirely due to \(Ca^{2+}\) influx through VDCC and SOC channels (23, 24). In the present study, to evaluate the participation of tyrosine kinase (TK) activity in the mechanism of muscle \([Ca^{2+}]_i\) regulation by \(1a,25(OH)_2D_3\), we first examined the effect of TK inhibition on the hormone-generated variations in intracellular \(Ca^{2+}\). Pretreatment of myoblasts with the tyrosine kinase inhibitors genistein (50–100 \(\mu M\)) and herbimycin (10–50 \(\mu M\)) completely prevented any subsequent response to \(1a,25(OH)_2D_3\) (Fig. 2A). The effects of both inhibitors on hormone-Ca\(^{2+}\) responses are likely to be due to suppression of TK activity. At the concentrations employed or higher (up to 370 \(\mu M\)), genistein has been previously shown not to alter cAMP-dependent kinase, protein kinase C (PKC), and phosphorylase kinase in other cell types (49–51). Moreover, daidzein, an inactive analog of genistein, at concentrations as high as 100 \(\mu M\) did not block the increase in muscle \([Ca^{2+}]_i\), caused by \(1a,25(OH)_2D_3\) (153 ± 10 and 162 ± 7 nm, for \(1a,25(OH)_2D_3\)-treated cells in the absence and presence of daidzein, respectively, at the peak of the \([Ca^{2+}]_i\) response; basal values were 98 ± 11 nm). Herbimycin inhibits protein-tyrosine kinases with higher selectivity than genistein (52, 53). The addition of either inhibitor to the medium after the plateau phase of sterol-dependent changes in \([Ca^{2+}]_i\), which had been reached did not affect further intracellular Ca\(^{2+}\) levels (Fig. 2B).

The effect of tyrosine phosphatase inhibition on the muscle cell \([Ca^{2+}]_i\) response to \(1a,25(OH)_2D_3\) was assayed using sodium orthovanadate (vanadate). In the presence of \(Ca^{2+}\), in the extracellular medium, 1 mM vanadate alone caused a more gradual increase in \([Ca^{2+}]_i\) than the hormone, which reached a plateau level (1.5–2-fold above basal values) 3 min after its addition, whereas no changes were detected when a \(Ca^{2+}\)-free medium was used (Fig. 3A). These observations suggest that in skeletal muscle cells, inhibition of tyrosine phosphatase activity promotes influx of \(Ca^{2+}\) from the extracellular milieu but not mobilization from endogenous stores. More important, the addition of vanadate to the sustained phase of the \(1a,25(OH)_2D_3\) \([Ca^{2+}]_i\) response produced no modification in the level of \(Ca^{2+}\) influx (Fig. 3B). Conversely, adding the sterol to the medium after the vanadate response reached the steady state had no effect on such a response (data not shown). Besides its effects on tyrosine phosphatases, vanadate has been shown to inhibit the \(Ca^{2+}\)-ATPase of plasma membrane (54). However, the possibility that the vanadate-induced increase in muscle cytosolic \(Ca^{2+}\) may be due to \(Ca^{2+}\)-ATPase inhibition is unlikely under our experimental conditions as genistein pretreatment of cells markedly reduced the vanadate-dependent increase in \([Ca^{2+}]_i\) (data not given).

To determine whether tyrosine phosphorylation of skeletal muscle cell proteins is modulated by the steroid hormone, cultured chick muscle cells were briefly (1 min) incubated with \(1a,25(OH)_2D_3\) (0.1–10 nm). As shown in Fig. 4, immunoprecipitation and Western blot analysis of cell lysates with a polyclonal antiserum reactive with phosphotyrosine residues revealed that the hormone causes a rapid increase in tyrosine phosphorylation of various cellular proteins. The effects of \(1a,25(OH)_2D_3\) were concentration-dependent, with maximal stimulation achieved at 1 nm. Significant changes in phosphotyrosine-containing proteins of relative molecular masses of 42–44, 65, and 127 kDa were observed in response to the hormone. Proteins of 140 and 20 kDa were also tyrosine-phosphorylated but to a lesser extent. The \(1a,25(OH)_2D_3\)-induced increment of protein phosphorylation could be suppressed by the tyrosine kinase inhibitor genestein (50–100 \(\mu M\)).

MAPK or extracellular signal-regulated kinase consists of...
42- and 44-kDa isoforms and requires both tyrosine and threonine phosphorylation for activation (55). To explore the possibility that 1a,25(OH)2D3 phosphorylates MAP kinase in muscle cells, the membranes from the experiments of Fig. 4 were stripped and reprobed with anti-phospho-MAP kinase antibody, which recognizes both the 42- and 44-kDa species of active phosphorylated MAP kinase. As shown in Fig. 5A, MAPK co-migrated with the tyrosine-phosphorylated bands at an estimated molecular mass of 42/44 kDa. Marked increases in phosphorylation could be detected after treatment with 1a,25(OH)2D3; the hormone effects on enzyme activity fell between 2 and 10 min to control levels. Similarly to MAP kinase phosphorylation, the 1a,25(OH)2D3-dependent increase in enzyme activity was completely abolished by the tyrosine kinase inhibitor genistein (Fig. 7B).

As revealed by the experiments of Fig. 4, one of the proteins that underwent a significant 1a,25(OH)2D3-dependent increase in tyrosine phosphorylation had a relative molecular mass of 127 kDa, which matches that of PLCγ. This isoform of polyphosphoinositide PLC is activated and associates to membranes by tyrosine phosphorylation (58, 59). To identify this macromolecule as PLCγ, lysates from muscle cells incubated with 1 nM 1a,25(OH)2D3 for 0.5–10 min were immunoprecipitated with anti-PLCγ antibody followed by anti-phosphotyrosine immunoblotting. A marked stimulation (1.5–2-fold) in the band of 127 kDa by hormone treatment was observed (Fig. 8).
In view of the role of MAPK in the regulation of cellular growth, studies were carried out to test whether the observed activation of MAPK by 1α,25(OH)2D3 was involved in the mitogenic effects of the hormone in proliferating skeletal muscle cells. Fig. 9 shows that both genistein (100 μM) and compound PD98059 (10 μM), which inhibits MAPK activation by the dual MAPK kinase MEK, effectively blocked the increase in myoblast DNA synthesis caused by 1 nM 1α,25(OH)2D3 during a 6–24-h treatment interval.

It has been previously shown that the proliferative effects of 1α,25(OH)2D3 in muscle cells are accompanied by enhanced mRNA levels of the nuclear proto-oncoprotein c-myc (29), known to induce the expression of genes involved in cell growth stimulation. There is evidence indicating that c-myc function may be regulated by phosphorylation (60, 61). We attempted to determine whether 1α,25(OH)2D3 stimulates tyrosine phosphorylation of c-myc, considering that another protein whose phosphorylation is highly specific anti-c-myc monoclonal antibody followed by Western blotting with anti-phosphotyrosine antibody (see Fig. 4), the membranes were stripped and re-probed with anti-phospho-MAP kinase antibody as described under “Experimental Procedures.” A, representative immunoblot. B, quantification by scanning volumetric densitometry of blots from three independent experiments; averages ± S.D. are given. *, p < 0.001.

In order to ascertain whether 1α,25(OH)2D3-induced tyrosine phosphorylation of c-myc has been already determined, lysates from muscle cells incubated with 1α,25(OH)2D3 for 1–10 min were immunoprecipitated with a highly specific anti-c-myc monoclonal antibody followed by Western blotting with anti-phosphotyrosine antibody. In agreement with the results of Fig. 4, c-myc appeared as a band ranging between 64 and 67 kDa in several independent experiments (average ± S.D. = 65.1 ± 1.3), its phosphorylation being markedly increased by 1α,25(OH)2D3 with respect to basal levels, e.g. 10-fold at 1 min and 50-fold after 5 and 10 min of sterol exposure, respectively (Fig. 10). In separate experiments, it was observed that pretreatment of muscle cells with the Src inhibitor PP1, both at 10 and 50 μM, completely suppressed hormone-dependent tyrosine phosphorylation of c-myc, whereas compound PD98059 was without effects (data not shown).

DISCUSSION

The results of the present investigation provide the first direct evidence involving TK activity in the regulation of intracellular Ca2+ homeostasis by 1α,25(OH)2-vitamin D3. In colonocytes, this has been only indirectly suggested by the finding that tyrosine phosphorylation mediates sterol activation of PLCγ (62), known to increase [Ca2+]i, via inositol 1,4,5-trisphosphate generation. In our study, pretreatment of chick skeletal muscle cells with the TK inhibitors genistein and herbimycin abolished both the transient and sustained phases of the 1α,25(OH)2D3 [Ca2+]i response, which reflect mainly Ca2+ release from the sarcoplasmic reticulum and extracellular Ca2+ influx, respectively (11, 17, 23). Furthermore, vandate, which inhibits protein-tyrosine phosphatases, also caused an increase in [Ca2+]i. Of relevance, no additional increase in Ca2+ influx could be observed by adding 1α,25(OH)2D3 or vandate at the plateau level of the [Ca2+]i response induced by either agent. The lack of effects of genistein and herbimycin, added when the sustained phase in [Ca2+]i, has been already
eral muscle cells were incubated with 1 nM 1α,25(OH)2D3, followed by anti-phosphotyrosine (anti-P-Tyr) immunoprecipitation and MAPK activity of the immunoprecipitate was measured using myelin basic protein as a substrate as described under “Experimental Procedures.” Results are the average of three independent experiments ± S.D. *p < 0.001; **p < 0.005.

FIG. 7. Stimulation of chick skeletal muscle cell MAPK activity by 1α,25(OH)2D3. A, time course. Muscle cells were treated with 1 nM 1α,25(OH)2D3 for 0.5 ± 10 min. B, myoblasts were treated with 1 nM 1α,25(OH)2D3 for 1 min in the presence or absence of 100 μM genistein. Cell lysates were immunoprecipitated with anti-phospho-MAPK antibody, and MAPK activity of the immunoprecipitate was measured using myelin basic protein as a substrate as described under “Experimental Procedures.” Results are the average of three independent experiments performed in duplicate ± S.D. *, p < 0.001; **, p < 0.005.

FIG. 8. Stimulation of PLCγ tyrosine phosphorylation by 1α,25(OH)2D3 in skeletal muscle cells. Cultured chick embryo skeletal muscle cells were incubated with 1 nM 1α,25(OH)2D3 for the indicated times. The cells were then lysed and immunoprecipitated with anti-PLCγ antibody. The immunoprecipitate was analyzed by SDS-PAGE followed by anti-phosphotyrosine (anti-P-Tyr) immunoblotting as described under “Experimental Procedures.” A representative immunoblot from three independent experiments is shown.

FIG. 9. 1α,25(OH)2D3-induced DNA synthesis in skeletal muscle cells is suppressed by genistein and MAPK kinase (MEK) inhibitor PD98059. Chick embryo skeletal muscle cells cultured for 24 h (myoblasts) were incubated for 6 to 24 h with or without 1 nM 1α,25(OH)2D3 in the presence or absence of PD98059 (10 μM) or genistein (100 μM). DNA synthesis was measured by [3H]thymidine incorporation as described under “Materials and Methods.” Results are the average of four independent experiments ± S.D. ***, p < 0.001; *, p < 0.05.

triggers 1α,25(OH)2D3, suggests that once the TK-dependent Ca2+-signaling mechanism is activated by the hormone, subsequent events unrelated to protein tyrosine phosphorylation are responsible for keeping muscle cell cytosolic Ca2+ levels elevated.

The fact that pretreatment of muscle cells with genistein and herbimycin completely suppressed the changes in intracellular Ca2+ induced by 1α,25(OH)2D3 indicates that tyrosine kinases mediate hormone stimulation of Ca2+ influx both through voltage-dependent and store-operated calcium channels. Sensitivity to TK inhibitors has been previously observed in various cell types with Ca2+-mobilizing agonists other than 1α,25(OH)2D3 for either the VDCC (33, 34, 63, 64) or SOC channel (35–37)-mediated Ca2+ entry. The finding that 1α,25(OH)2D3 rapidly (within 1 min) stimulates tyrosine phosphorylation of PLCγ (Fig. 8) strongly suggests that activation of this PLC isoform mediates, at least in part, inositol 1,4,5-trisphosphate-dependent Ca2+ release from inner stores, causing in turn the entry of extracellular Ca2+ through SOC channels. In addition, enhancement of PLCγ activity by 1α,25(OH)2D3 may result, through diacylglycerol generation, in stimulation of PKC, which mediates sterol regulation of muscle cell VDCC (20). We have recently shown that hormone treatment of skeletal muscle cells induces a very fast increase in the activity of the non-receptor tyrosine kinase Src (65), a proximate activator of PLCγ (66, 67).

Altogether, these results suggest that protein tyrosine phosphorylation is a previously unrecognized mechanism that functions in concert with other membrane-signaling pathways (11, 16) to increase 1α,25(OH)2D3-dependent intracellular Ca2+ levels in skeletal muscle cells. Further investigations are required to elucidate how interaction of 1α,25(OH)2D3 at its primary site of action couples to the TK-mediated release of Ca2+ from intracellular stores and the influx through membrane Ca2+ channels. The intracellular vitamin D receptor itself may mediate the fast enhancement of tyrosine kinase activity in muscle cells. We have recently shown that 1α,25(OH)2D3 significantly increases tyrosine phosphorylation of the vitamin D receptor, which is paralleled by association to and stimulation by tyrosine dephosphorylation of the non-receptor tyrosine kinase Src (65). The activation of Src and, in turn, of PLCγ by 1α,25(OH)2D3 has been shown in rat coloconocytes, but the intervention of the vitamin D receptor in the hormon e effects was not demonstrated (62). Alternatively, we proposed (65) that a possible mechanism by which 1α,25(OH)2D3 stimulates Src activity in muscle cells requires binding of 1α,25(OH)2D3 to its cognate receptor, thus inducing a conformational change on this protein, which is then sensed by the receptor-associated Src.

This study demonstrates in addition that tyrosine kinase activity also plays a key role in the stimulation of skeletal muscle cell division by 1α,25(OH)2D3. Anti-phosphotyrosine immunoblot analysis revealed that 1α,25(OH)2D3 rapidly stimulates tyrosine phosphorylation of various muscle cell proteins, among which three major targets of the hormone of 42/44, 65,
and 127 kDa could be identified as the growth-related proteins MAP kinase (extracellular signal-regulated kinase 1/2), c-myc, and PLCγ, respectively, on the basis of their immunoreactivity with corresponding selective antibodies. In the case of MAPK, the increase in phosphorysine content by 1α,25(OH)2D3 was accompanied by an elevation of its enzymatic activity. In line with these observations, it has been recently reported that 1α,25(OH)2D3 induces a rapid stimulation of MAP kinase phosphorylation in promyelocytic NB4 leukemia cells (40) and enterocytes (41).

Stimulation of the MAP kinase 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 (31, 32, 68–70). There is evidence that the effect of 1α,25(OH)2D3 on the MAPK pathway in chick skeletal muscle cells involves a rapid increase in Src activity (65). Also, PKC partially mediates hormone stimulation of MAPK (71). This is in keeping with previous studies involving PKC in 1α,25(OH)2D3 regulation of muscle intracellular calcium homeostasis and cell proliferation, provide a new basis for understanding abnormalities in muscle contractility and growth associated with various vitamin D-related disorders such as renal osteodystrophy, chronic renal failure, and osteomalacia.

Fig. 10. 1α,25(OH)2D3 phosphorylates the transcription factor c-myc in skeletal muscle cells. Chick embryo skeletal muscle cells cultured for 24 h (myoblasts) were exposed for 1–10 min to 1 nm 1α,25(OH)2D3 or vehicle ethanol. The cells were then lysed, immunoprecipitated with anti-c-myc antibody followed by Western blotting with anti-phosphotyrosine (anti-P-Tyr) antibody. A, representative immunoblot. B, quantification by scanning volumetric densitometry of blots from four independent experiments; averages ± S.D. are given. *, p < 0.001.

REFERENCES
1. Norman, A. W., Roth, J., and Oei, L. (1982) Endoocr. Rev. 3, 331–365
2. De Luca, H. F. (1988) FASEB J. 2, 224–236
3. Reichel, H., and Norman, A. W. (1989) Annu. Rev. Med. 40, 71–78
4. Manolagas, S. C., Huhtmyer P. G., and Yu, X-P. (1990) Kidney Int. 38, 9–16
5. Walters, M. R. (1990) Endoocr. Rev. 11, 719–764
6. Ekblom, K., Kirstein, L., and Astrum, K. E. (1964) Acta Med. Scand. 176, 493–497
7. Smith, R., and Stern, G. (1967) Brain 90, 593–602
8. Smith, R., and Stern, G. (1969) J. Neurol. Sci. 8, 511–520
9. Boland, R. L. (1969) Endoocr. Rev. 4, 225–448
10. Giuliani, D., and Boland, R. L. (1984) Cellul. Tissue Int. 36, 206–265
11. Boland, R. L., De Boland, A. R., Marzinzik, M., Santillan, G., Vazquez, G., and Zanello, S. (1995) Mol. Cell. Endoocr. 114, 1–8
12. Haussler, M. R. (1986) Annu. Rev. Nutr. 6, 527–562
13. Minghetti, P. P., and Norman, A. W. (1988) FASEB J. 2, 3043–3053
14. Lieberherr, M., Grosse, B., Duchampon, P., and Druke, R. (1989) J. Biol. Chem. 264, 52063–52066
15. Norman, A. W., Nemeere, Y., Zhou, L-X., Bishop, J. E., Lowe, K. E., Maimar, A. C., Collino, R. D., Taoka, T., Serezy, Y., and Farach-Carson, M. C. (1992) J. Steroid Biochem. Mol. Biol. 41, 231–240
16. De Boland, A. R., and Boland, R. L. (1994) Cell. Signal. 6, 717–724
17. Morelli, S., De Boland, A. R., and Boland, R. L. (1990) Biochim. J. 288, 675–679
18. De Boland, A. R., Morelli, S., and Boland, R. L. (1991) Biochim. Biophys. Acta 1257, 274–278
19. Vazquez, G., and De Boland, A. R. (1996) Biochim. Biophys. Acta 1310, 157–162
20. Morelli, S., Boland, R., and De Boland, A. R. (1996) Mol. Cell. Endoocr. 122, 207–211
21. Vazquez, G., De Boland, A. R., and Boland, R. L. (1997) Biochim. Biophys. Acta 23, 125–128
22. Vazquez, G., De Boland, A. R., and Boland, R. L. (1997) Biochim. Biophys. Acta 23, 562–565
23. Vazquez, G., De Boland, A. R., and Boland, R. (1998) J. Biol. Chem. 273, 39065–39069
24. Boland, R., De Boland, A. R., Vazquez, G., Santillan, G., and Monje, P. (1997) in Vigny's D Chemistry, Biology, and Clinical Applications of the Steroid Hormone (Norman, A. W., Bouillon, R., and Thomasset, M., eds) pp. 361–368, University of California Press, Riverside, CA
25. Wehling, M. (1997) Annu. Rev. Physiol. 59, 655–693
26. Menon, I., and Farach-Carson, M. C. (1998) Biochim. Biophys. Acta 248, 443–449
27. Bellido, T., Morelli, S., Fernandez, L. M., and Boland, R. (1995) Mol. Cell. Endoocr. 90, 231–238
28. Drzystan, L., De Boland, A. R., and Boland, R. L. (1989) Biochim. Biophys. Acta 104, 112–119
29. Capiati, D. A., Telzer-Ionin, M. T., and Boland, R. L. (1999) Mol. Cell. Endoclor. 153, 39–45
30. Neary, J. T. (1997) News Physiol. Sci. 12, 286–293
31. Selbie, L. A., and Hill, S. J. (1997) Trends Pharmacol. Sci. 19, 87–93
32. Cataldi, M., Tagliatela, M., Guerriero, S., Amoroso, S., Lombardi, G., del Rezzo, G., and Annunziato, L. (1996) J. Biol. Chem. 271, 9441–9446
33. Hatakeyama, M., Nukohhadhay, D., Goyal, R. K., and Akbarali, H. L. (1996) Annu. J. Physiol. 270, C1780–C1789
34. Lee, K. M., Tocas, K., and Villereal, M. L. (1998) J. Biol. Chem. 268,
