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Activation of RAF-1 through Ras and Protein Kinase Cα Mediates 1α,25(OH)$_2$-Vitamin D$_3$ Regulation of the Mitogen-activated Protein Kinase Pathway in Muscle Cells*

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We have previously shown that stimulation of proliferation of avian embryonic muscle cells (myoblasts) by 1α,25(OH)$_2$-vitamin D$_3$ (1α,25(OH)$_2$D$_3$) is mediated by activation of the mitogen-activated protein kinase (MAPK; ERK1/2). To understand how 1α,25(OH)$_2$D$_3$ up-regulates the MAPK cascade, we have investigated whether the hormone acts upstream through stimulation of Raf-1 and the signaling mechanism by which this effect might take place. Treatment of chick myoblasts with 1α,25(OH)$_2$D$_3$ (1 nM) caused a fast increase of Raf-1 serine phosphorylation (1- and 3-fold over basal at 1 and 2 min, respectively), indicating activation of Raf-1 by the hormone. These effects were abolished by preincubation of cells with a specific Ras inhibitor peptide that involves Ras in 1α,25(OH)$_2$D$_3$ stimulation of Raf-1. 1α,25(OH)$_2$D$_3$ rapidly induced tyrosine de-phosphorylation of Ras-GTPase-activating protein, suggesting that inhibition of Ras-GTP hydrolysis is part of the mechanism by which 1α,25(OH)$_2$D$_3$ activates Ras in myoblasts. The protein kinase C (PKC) inhibitors calphostin C, bisindolylmaleimide I, and Ro 318220 blocked 1α,25(OH)$_2$D$_3$-induced Raf-1 serine phosphorylation, revealing that hormone stimulation of Raf-1 also involves PKC. In addition, transfection of muscle cells with an antisense oligodeoxynucleotide against PKCα mRNA suppressed serine phosphorylation by 1α,25(OH)$_2$D$_3$. The increase in MAPK activity and tyrosine phosphorylation caused by 1α,25(OH)$_2$D$_3$ could be abolished by Ras inhibitor peptide, compound PD 98059, which prevents the activation of MEK by Raf-1, or incubation of cell lysates before 1α,25(OH)$_2$D$_3$ exposure with an anti-Raf-1 antibody. In conclusion, these results demonstrate for the first time in a 1α,25(OH)$_2$D$_3$ target cell that activation of Raf-1 via Ras and PKCα-dependent serine phosphorylation plays a central role in hormone stimulation of the MAPK-signaling pathway leading to muscle cell proliferation.

Raf-1 was discovered as the first member of a cytoplasmatic family of serine/threonine kinases and plays a crucial role in the activation of the classical cytoplasmatic-signaling cascade that is involved in the regulation of cellular proliferation, differentiation, and apoptosis (1). Activation of Raf-1 downstream of protein-tyrosine kinase receptors is mediated by the Ras GTP-binding proteins, which are required for stimulation of Raf-1 kinase activity (2, 3). Raf-1 then phosphorylates and activates the mitogen-activated protein kinase kinase, also known as MEK[1] (4), initiating a protein kinase cascade that follows with the phosphorylation and activation of the extracellular signal-regulated mitogen-activated protein (MAP) kinase isoforms ERK1 and ERK2 (5). Upon activation, MAP kinase (MAPK) is translocated from the cytoplasm into the nucleus (6), where it phosphorylates transcription factors (7) and culminates in proliferation or differentiation of a variety of cell types.

With an estimated molecular mass of 74 kDa, Raf-1 possesses three conserved regions, CR1, CR2, and CR3, that are embedded in variable regions. The CR1 and CR2 domains are part of the regulatory N-terminal half of the Raf-1 protein, whereas CR3 forms the C-terminal kinase domain (1). The molecular mechanism of Raf-1 activation, however, is still unclear. Although Raf-1 binds directly to the effector domain of activated GTP-bound Ras proteins, this interaction does not appear to stimulate Raf-1 kinase activity (8). Rather, it seems that the role of the Ras-Raf interaction is to recruit Raf-1 to the plasma membrane, where it can be activated by membrane lipids or other protein kinases, the nature of which remains to be determined (9, 10). Phosphorylation of Raf on both tyrosine and serine/threonine residues is required for enzymatic activity (11). It has been shown that the Src protein-tyrosine kinase can activate Raf by phosphorylation on Tyr-340 and Tyr-341 (12, 13). However, mutant Raf proteins in which Tyr-340 and Tyr-341 have been changed to Asp can still be activated after membrane association, indicating that an additional mechanism(s) of Raf activation exists (13). Among the mechanisms involved, there is evidence for the operation of both protein kinase C (PKC)-dependent and PKC-independent pathways of Raf activation in response to agonists (14).

The 13 members of the PKC family can be grouped into three major classes of Ca^{2+}-dependent classical PKCs, Ca^{2+}-independent, novel PKCs, and Ca^{2+}- and lipid-independent, atypical PKCs. There is a fourth PKC subgroup consisting of PKCμ (15). Previous reports showed that classical and novel PKCs activate the MAP kinase pathway at the level of Raf-1, whereas atypical PKCs activate MEK by an independent mechanism (16, 17). One of the classical PKCs, PKCα can directly activate

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[1] The abbreviations used are: MEK, mitogen-activated protein (MAP) kinase (MAPK) kinase; ERK, extracellular signal-regulated kinase; PKC, protein kinase C; 1α,25(OH)$_2$D$_3$, 1α,25-dihydroxyvitamin D$_3$; DMEM, Dulbecco's modified Eagle's medium; GAP, GTPase-activating protein; ODN, oligodeoxynucleotide.

Raf-1 by serine phosphorylation of Raf-1 (18).

The steroid hormone 1α,25-dihydroxy-vitamin D_3 ($1\alpha,25(OH)_2D_3$) triggers responses in muscle cells both through a nuclear receptor-mediated mechanism that promotes gene transcription (19) and a fast non-genomic mode of action independent of new RNA and protein synthesis (20, 21). In previous work we have demonstrated that the steroid hormone rapidly stimulates in skeletal muscle cells the phosphorylation and activity of the MAP kinase isoforms ERK1 and ERK2 and have implicated the MAPK cascade in hormone control of myoblast proliferation (22). Moreover, initial investigations on the mechanisms underlying $1\alpha,25(OH)_2D_3$ stimulation of the muscle cell (myoblast) MAPK pathway revealed that PKC and Ca^{2+} are two upstream activators mediating the hormone effect (23). In addition, it has been shown that $1\alpha,25(OH)_2D_3$ enhancement of myoblast proliferation correlates to increased PKCα expression, whereas decreased PKCα levels are observed during the subsequent activation of muscle cell differentiation by the hormone (24). Furthermore, inhibition of PKCα expression by using antisense oligonucleotide technology resulted in a significant decrease of culture cell density and DNA synthesis, clearly showing that this isozyme is involved in signaling cascades that promote muscle cell proliferation (25).

The upstream-signaling pathway that leads to activation of the Ras/Raf-1/MAPK (ERK1/2) cascade by $1\alpha,25(OH)_2D_3$ remains incompletely understood, and direct evidence on the participation of any of the PKC isoforms is lacking. In view of the information discussed above, we have investigated the role of Ras as well as PKCα in Raf 1 activation.

MATERIALS AND METHODS

Chemicals—$1\alpha,25(OH)_2D_3$ was kindly provided by Hoffmann-La Roche. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, specific Ras inhibitory peptide (VPPPVPPRRR), and protein A-Sepharose were from Sigma. Lipofectin was from Invitrogen. Sense and antisense oligodeoxynucleotides were synthesized by the DNAgency (Malvern, PA). Rabbit polyclonal anti-phosphoserine antibody was obtained from Upstate Biotechnology Inc. (Lake Placid, NY). Anti-Raf-1 monoclonal antibody and anti-(phospho)-active MAP kinase antibody (reactive against p42 and p44 isoforms) were from Promega (Madison, WI). Anti-Ras-GTPase-activating protein (GAP) antibody and donkey anti-goat IgG antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary antibody goat anti-rabbit horseradish peroxidase-conjugated IgG and the Super Signal CL-HRP substrate system for enhanced chemiluminescence (ECL) were obtained from Amersham Biosciences. [γ-^{32}P]ATP (3,000 Ci/mmol) was from PerkinElmer Life Sciences. The compounds PD 98059, Ro 318220, calphostin C, and bisindolylmaleimide I were from Calbiochem. All other reagents 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 0.06% trypsin for 30 min essentially as previously described (26). 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 unadsorbed cells were seeded at an appropriate density (120,000 cells/cm^2) in Petri dishes (100-mm diameter) and cultured at 37 °C under a humidified atmosphere (air 95%, 5% CO_2). Under these conditions, myoblasts divide within the first 48 h and at day 4 become differentiated into myotubes expressing both biochemical and morphological characteristics of adult skeletal muscle fibers (27). Cells cultured for 2 days (proliferative stage) were used for treatments.

Immunoprecipitation—After $1\alpha,25(OH)_2D_3$ or vehicle (ethanol, $<$ 0.01%) treatment, muscle cells were lysed (15 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 aprotinin, 0.25% sodium deoxycholate, and 1% Nonidet P-40 and homogenized by sonication for 15 s. Insoluble material was pelleted in a microcentrifuge at 12,000 $\times$ *g* for 15 min. The protein content of the clear lysates was determined according to Lowry *et al.* (28). Aliquots (500–700 μg of protein) were incubated overnight at 4 °C with anti-Raf-1 or anti-Ras-GAP antibodies followed by precipitation of the complexes with protein A conjugated with Sepharose. The immune complexes were washed 4 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-100m and 1% Nonidet P-40) and a final wash in cold phosphate-buffered saline.

Blockade with Antibodies—Insoluble material of lysate proteins was pelleted in a microcentrifuge at 12,000 $\times$ *g* for 15 min. The protein content of the clear lysates was determined according to Lowry *et al.* (28). Aliquots (500 μg of protein) were incubated with anti-Raf-1 or anti-goat IgG antibody on an ice bath with shaking for 10 min. Then the lysates were exposed to 1 nM $1\alpha,25(OH)_2D_3$ or vehicle (ethanol $<$ 0.01%) for 1 min. The treatments were stopped by adding Laemmli sample buffer (29).

SDS-PAGE and Immunoblotting—Immunoprecipitated proteins (or lysate proteins) dissolved in Laemmli sample buffer were separated on SDS-polyacrylamide (8%) gels (29) 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 5% dry milk. Membranes were subjected to immunoblotting using anti-PKCα, anti-phosphoserine, or anti-phosphotyrosine antibodies. Next the membranes were washed 3 times in TBST, incubated in TBST containing 1% dry milk with a 1:10,000 dilution of peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies for 1 h at room temperature, and washed 3 additional times with TBST. The membranes were then visualized using an enhanced chemiluminescent technique (ECL, Amersham Biosciences) according to the manufacturer's instructions. Images were obtained with a model GS-700 Imaging Densitomer from Bio-Rad by scanning at 600 dots per inch and printing at the same resolution. Bands were quantified using the Molecular Analyst program (Bio-Rad).

To strip the membranes for reprobing with anti-Raf-1, anti Ras-GAP, or anti-ERK1/2 antibodies, the membranes were washed for 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 membranes were again blocked and blotted as described above.

Measurement of MAP Kinase Activity—Muscle cells were pretreated with PD 98059 (10 μM, 10 min) or Ras inhibitor peptide (25 μM, 10 min) and then exposed for 1 min with 1 nM $1\alpha,25(OH)_2D_3$ or vehicle ($<$ 0.01% ethanol) at 37 °C. Lysates were prepared followed by immunoprecipitation of MAP kinase (p42 and p44) as described above. After 3 washes with immunoprecipitation buffer and 2 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 phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, and 20 μg/ml aprotinin), 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 into ice-cold 75 mM H_3PO_4, washed (1 $\times$ 5 min, 3 $\times$ 20 min), and counted in a scintillation counter.

Cell Transfection—Transfection with oligodeoxynucleotides (ODNs) using Lipofectin was performed according to the manufacturer's instructions. ODNs were incubated with Lipofectin in DMEM for 15 min at room temperature. Plates of subconfluent cells were washed to remove serum before the addition of ODN-Lipofectin mixtures, and incubation was performed for 4 h at 37 °C. The ODN solution was removed, DMEM was added, and the plates were placed into a metabolic incubator for an additional period of 20 h. Control treatments included DMEM or Lipofectin only. Dose- and time-response studies for Lipofectin and ODNs were previously performed to establish optimum conditions for the effective blockade of PKCα expression (25). The following ODN sequences with phosphorothioate linkages throughout the entire ODN molecule were used: antisense-PKCα (AS), 5′-CATGGTYC-CCCCCAACCACC-3′, Y = T or C (antisense sequence against 20 nucleotides upstream of the AUG codon); sense PKCα (S), 5′-GGTGGT-TGGGGGGRACCATG-3′, R = A or G (25). Each antisense oligonucleotide was used at a final concentration of 5 μM. These antisense sequences showed no homology to any DNA in the GenBank™ except PKCα (human, rat, mouse, and rabbit species). Cell death in cultures under control and treatment (ODNs) conditions was measured by trypan blue staining.

Statistical Analysis—Statistical significance of the data was evaluated using Student's *t* test (30), and probability values below 0.05 ($p < 0.05$) were considered significant. Results are expressed as the means $\pm$ S.D. from the indicated set of experiments.

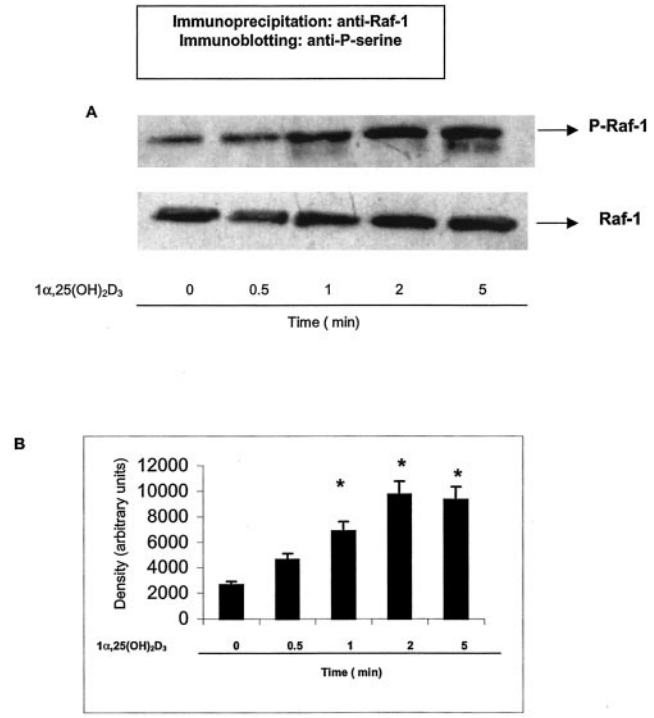


FIG. 1. **Time course of $1\alpha,25(OH)_2D_3$ stimulation of Raf-1 serine phosphorylation.** Chick skeletal muscle cells were incubated in the presence of $1\alpha,25(OH)_2D_3$ (1 nM) or vehicle (ethanol <0.01%) for the indicated times. Immunoprecipitation of Raf-1 and immunoblotting with an anti-phosphoserine (*anti-P-serine*) antibody were carried out in cell lysates as detailed under "Materials and Methods." *A*, representative immunoblot. *B*, quantification by scanning volumetric densitometry of blots from three independent experiments performed in duplicate; averages ± S.D. are given. *, $p < 0.01$, with respect to the control.

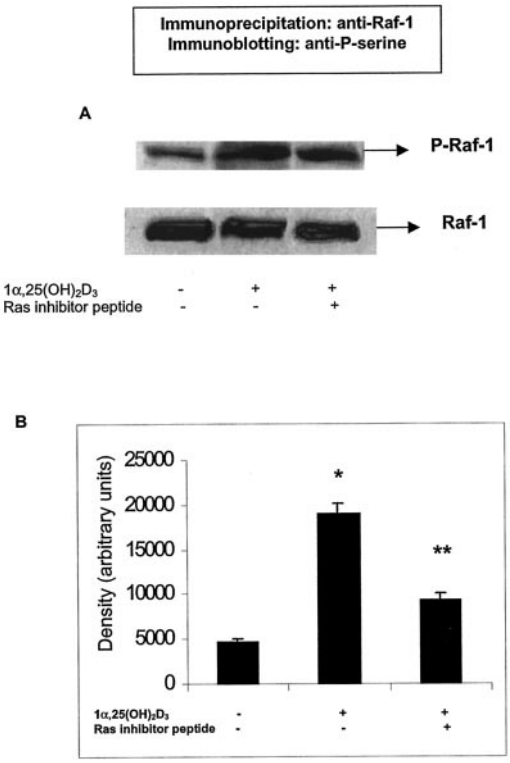


FIG. 2. **$1\alpha,25(OH)_2D_3$-induced serine phosphorylation (*P*) of Raf-1 is suppressed by Ras inhibitor peptide.** Chick skeletal muscle cells were preincubated for 2 h with a specific Ras inhibitor peptide (25 μM) and then exposed to 1 nM $1\alpha,25(OH)_2D_3$ for 2 min. After cell lysis, comparable aliquots were immunoprecipitated with an anti-Raf-1 antibody followed by Western blotting with anti-phosphoserine antibody as described under "Materials and Methods." *A*, representative immunoblot. *B*, quantification by scanning volumetric densitometry of blots of three independent experiments performed in duplicate; averages ± S.D. are given. *, $p < 0.01$, with respect to basal; **, $p < 0.05$ with respect to $1\alpha,25(OH)_2D_3$ stimulation.

RESULTS AND DISCUSSION

To understand how the steroid hormone $1\alpha,25(OH)_2D_3$ controls the MAP kinase cascade in skeletal muscle cells, it is essential to identify the molecules that participate in the cellular sequence of events involved in the signaling pathway of this steroid hormone. As in other cell types, Raf-1 and MEK belong to the MAP kinase cascade that leads to muscle cell proliferation (31, 32). As a major step in this direction, we report here for the first time that $1\alpha,25(OH)_2D_3$ stimulation of the MAPK (ERK1/2) pathway in skeletal muscle cells involves at least in part rapid activation of Raf-1 and provide information on the mechanism of action by which this hormone-regulated event takes place.

The complex process of Raf activation is still incompletely understood. Existing data suggest that activation of Raf-1 engages multiple factors and steps (9, 10), and phosphorylation of Raf-1 on Ser 338 and Tyr-341 is a critical step in this process (11). To evaluate whether the serine-threonine kinase Raf-1 is part of the $1\alpha,25(OH)_2D_3$-signaling mechanism in chick muscle cells, we first investigated the effect of the steroid hormone on Raf-1 serine phosphorylation. To that end, muscle cells were exposed to 1 nM $1,25(OH)_2D_3$ (0.5–5 min), and cell lysates were immunoprecipitated with a highly specific anti-Raf-1 monoclonal antibody followed by immunoblotting with anti-phosphoserine antibody. As shown in Fig. 1, $1\alpha,25(OH)_2D_3$ caused a time-dependent increase in Raf-1 phosphorylation in muscle cells. The stimulation of Raf-1 serine phosphorylation could be detected already at 30 s, increased 1-fold over basal at 60 s, and reached a maximum after 2 min of hormone exposure (3-fold).

It has been reported that the small G protein Ras is the direct upstream activator of Raf, which in its GTP-bound activated form binds to the Ras binding domain (Raf-RBD) and recruits the inactive cytoplasmic Raf to the plasma membrane for activation (33). This binding induces a conformational change of Raf-1 that yields an opened structure suitable for phosphorylation by membrane-associated kinases. To study Ras-dependent changes in Raf activation induced by $1\alpha,25(OH)_2D_3$, muscle cells were preincubated for 2 h with a specific Ras inhibitor peptide, which blocks the association of Grb2 and Sos1 and, thus, the nucleotide exchange of Ras, avoiding its activation (34), followed by hormone treatment (1 nM, 2 min). As shown in Fig. 2, Ras inhibition abolished Raf-1 serine phosphorylation by $1\alpha,25(OH)_2D_3$, demonstrating that Ras is necessary for $1\alpha,25(OH)_2D_3$-Raf-1 activation in these cells. At the concentration used (25 μM), it is likely that sufficient amounts of the inhibitor peptide entered the cells by endocytosis to reach the K_d (25 nM) for its binding to Grb2 (35).

Ras proteins play a central role in control of cell proliferation, differentiation, and other cellular functions (36). They function by cycling between inactive GDP- and active GTP-bound forms. This molecular switch is mainly regulated by guanine nucleotide exchange factors, which catalyze an exchange of GTP for GDP, and by GAPs, which activate the intrinsic GTPase activity of Ras and, thus, convert Ras-GTP to Ras-GDP (37). It has been reported that tyrosine phosphorylation of Ras-GAPs on Tyr-460 allows rise of its GTPase activity (38), promoting Ras inactivation. Therefore, the tyrosine phosphorylation of Ras-GAP would promote Ras inactivation by Ras-GAP-induced hydrolysis of Ras-GTP bound. We next explored the effect of $1\alpha,25(OH)_2D_3$ on Ras-GAP tyrosine phosphorylation. As shown in Fig. 3, muscle cells treated with 1 nM

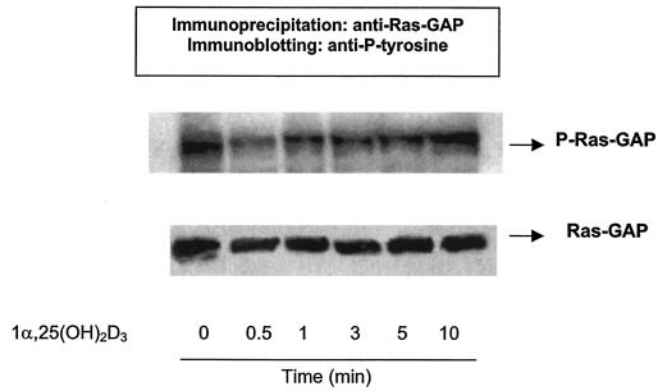
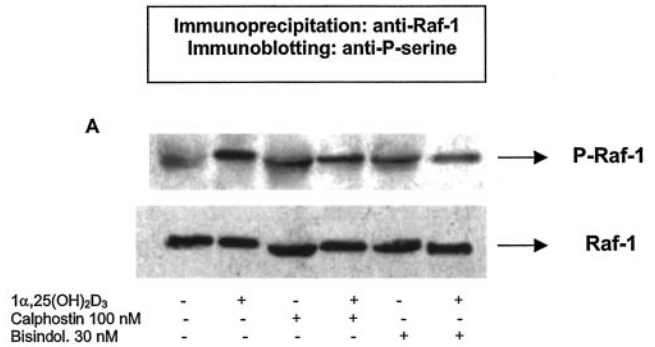


FIG. 3. **Transient inactivation of Ras-GAP by tyrosine dephosphorylation (*P*) in response to $1\alpha,25(OH)_2D_3$.** Lysates from chick muscle cells exposed to 1 nM $1\alpha,25(OH)_2D_3$ for the indicated times were immunoprecipitated with anti-Ras-GAP antibody followed by immunoblot analysis with anti-phosphotyrosine antibody as described under "Materials and Methods." A representative immunoblot from three independent experiments is shown. The blotted membranes were reprobed with anti-Ras-GAP antibody to evaluate the equivalence of Ras-GAP content among the different experimental conditions (*bottom panel*).



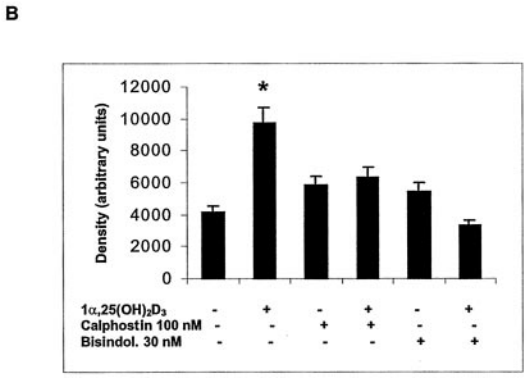
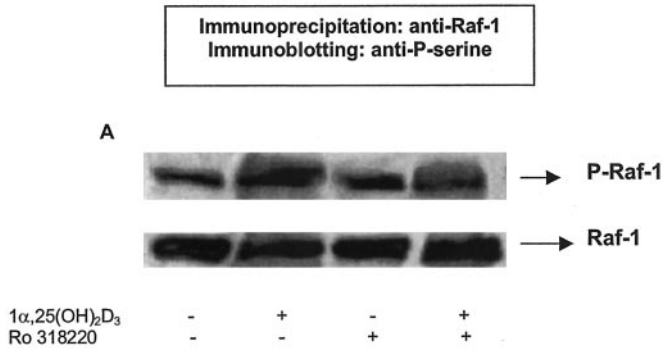


FIG. 4. **PKC inhibitors calphostin C and bisindolylmaleimide I suppress muscle cell Raf-1 serine phosphorylation (*P*) induced by $1\alpha,25(OH)_2D_3$.** Muscle cells were treated with 1 nM $1\alpha,25(OH)_2D_3$ for 2 min in the absence or presence of calphostin C (100 nM) or bisindolylmaleimide I (30 nM). The cells were then lysed and immunoprecipitated with anti-Raf-1 antibody followed by Western blotting with anti-phosphoserine antibody as described under "Materials and Methods." *A*, representative immunoblot. *B*, quantification by scanning volumetric densitometry of blots from three independent experiments performed in duplicate; averages ± S.D. are given. *, $p < 0.01$ with respect to basal and $1\alpha,25(OH)_2D_3$ stimulation in the presence of PKC inhibitors.

$1\alpha,25(OH)_2D_3$ showed a rapid and transient tyrosine de-phosphorylation of Ras-GAP, which was maximal at 0.5 min (−80%) and returned to basal level upon 10 min of hormone exposure.



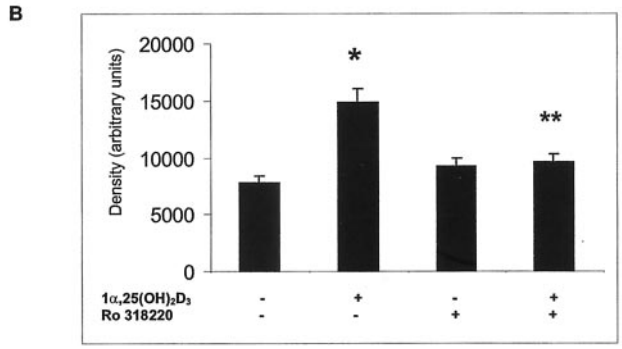


FIG. 5. **The PKC inhibitor Ro 318220 blocks the activation of Raf-1 induced by $1,25(OH)_2D_3$.** Muscle cells were treated with 1 nM $1,25(OH)_2D_3$ for 2 min in the absence or presence of Ro 318220 (200 nM). Then cells were lysed and immunoprecipitated with anti Raf-1 antibody followed by Western blotting with anti-phosphoserine (*anti-P-serine*) antibody as described under "Materials and Methods." *A*, representative immunoblot. *B*, quantification by scanning volumetric densitometry of blots from three independent experiments performed in duplicate; averages ± S.D. are given. *, $p < 0.01$, with respect to basal; **, $p < 0.05$ with respect to $1\alpha,25(OH)_2D_3$ stimulation.

This result suggests that inhibition of Ras-GTP hydrolysis is part of the mechanism by which $1\alpha,25(OH)_2D_3$ activates Ras in skeletal muscle cells.

PKC, discovered as a serine/threonine kinase (39), mediates intracellular responses to a variety of agonists including $1\alpha,25(OH)_2D_3$ (40). The direct phosphorylation of Raf-1 by PKC isoforms has been suggested as an activation mechanism of PKC on the Raf-1/ERK1/2-signaling pathway (16, 18). In previous investigations we have demonstrated that stimulation of the extracellular signal-regulated mitogen-activated protein kinases ERK1 and ERK2 by $1\alpha,25(OH)_2D_3$ is mediated by PKC and Ca^{2+} (23). These data prompted us to study whether PKC participates in Raf-1 serine phosphorylation induced by the hormone. To that end, muscle cells were pretreated with the PKC inhibitors calphostin C or bisindolylmaleimide I and stimulated with $1\alpha,25(OH)_2D_3$ (1 nM, 2 min) followed by immunoprecipitation of cell lysates with anti-Raf-1 antibody and then immunoblotting with anti-phosphoserine antibody. As shown in Fig. 4, Raf-1 serine phosphorylation was suppressed by either PKC inhibitor. Moreover, Ro 318220, a PKC specific inhibitor that blocks all PKC isoforms completely, abolished Raf-1 serine phosphorylation (Fig. 5) and further confirmed that hormone activation of Raf-1 is a PKC-dependent event. It has been shown that PKCα can directly phosphorylate and activate Raf-1 in other cell types (18). PKCα belongs to the family of conventional protein kinases that are Ca^{2+}-dependent. It is well recognized that when skeletal muscle cells are subjected to $1\alpha,25(OH)_2D_3$ stimulation, a rapid increase of intracellular Ca^{2+} (22), inositol trisphosphate, and diacylglyc-

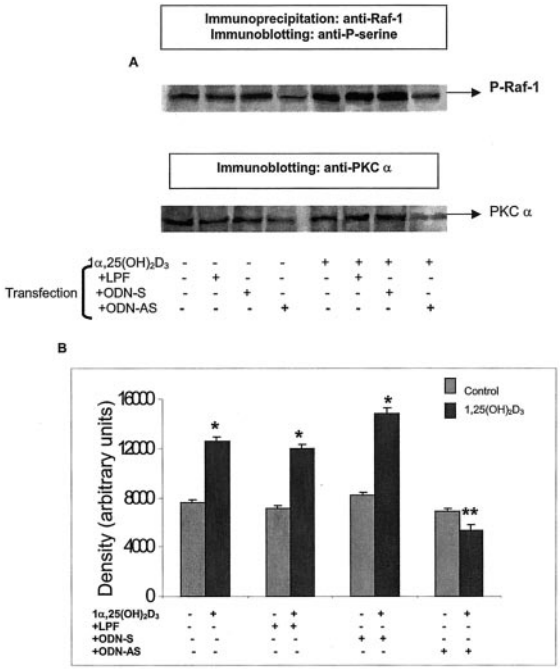


FIG. 6. **Effects of an antisense oligodeoxynucleotide against PKCα mRNA on $1,\alpha 25(OH)_2D_3$-induced Raf-1 serine phosphorylation.** Muscle cells were transfected with a sense (*ODN-S*) or an antisense (*ODN-AS*) oligodeoxynucleotide against PKCα mRNA or Lipofectin (*LPF*) to have a basal expression control. 48 h later the cells were exposed to 1 nM $1\alpha,25(OH)_2D_3$ for 2 min. Raf-1 was immunoprecipitated from cell lysates and then immunoblotted with anti-phosphoserine (*anti-P-serine*) antibody. *A*, *top panel*, representative blot showing changes in Raf-1 serine phosphorylation; *bottom panel*, representative blot showing the amount of PKCα expressed in the cells. *B*, quantification by scanning volumetric densitometry of top blot from four independent experiments performed in duplicate; averages ± S.D. are given. $p < 0.01$ (*) and $p < 0.05$ (**), with respect to the corresponding control.

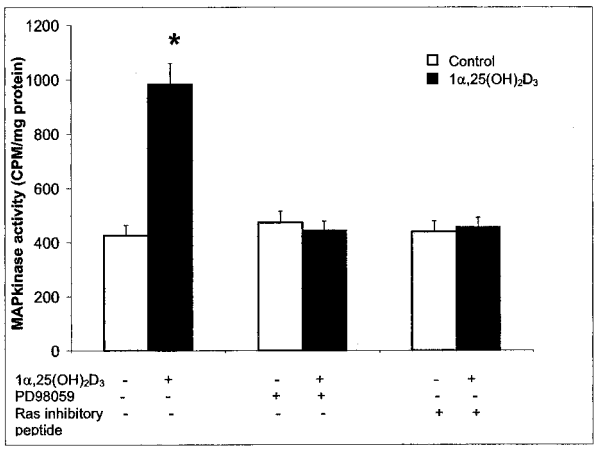


FIG. 7. **Stimulation of MAP kinase activity by $1\alpha,25(OH)_2D_3$ is mediated by Ras and MEK.** Cells were incubated for 1 min with $1\alpha,25(OH)_2D_3$ (1 nM) in the absence or presence of a MEK-specific inhibitor (PD 98059, 10 μM) or a Ras inhibitory peptide (25 μM). Immunoprecipitation of MAP kinase and assay of its activity using [γ-^{32}P]ATP and myelin basic protein as exogenous substrate were carried out in cell lysates as described under "Materials and Methods." Results are the average of three independent experiments performed in triplicate ± S.D. *, $p < 0.01$, with respect to basal and $1\alpha,25(OH)_2D_3$ stimulation in the presence of inhibitors.

erol (43) and activation of PKCα (44) occurs. To demonstrate that PKCα is involved in Raf-1 activation, we used antisense technology to block PKCα protein expression. Cultured muscle cells were transfected with an antisense oligodeoxynucleotide against 20 nucleotides upstream of the AUG codon of PKCα mRNA to suppress expression of its encoded protein. Under these conditions, phosphorylation of Raf-1 induced by $1\alpha,25(OH)_2D_3$ was fully abolished (Fig. 6*A*, *upper panel*). Suppression of PKCα expression was verified by immunoblotting of cell lysates with anti-PKCα antibody (Fig. 6*A*, *bottom panel*). Although we cannot rule out the possibility that other PKC isoforms may also contribute to Raf-1 serine phosphorylation, our results clearly show that PKCα is also a component of the mitogenic pathway leading to Raf-1 activation in response to $1\alpha,25(OH)_2D_3$ stimulation.

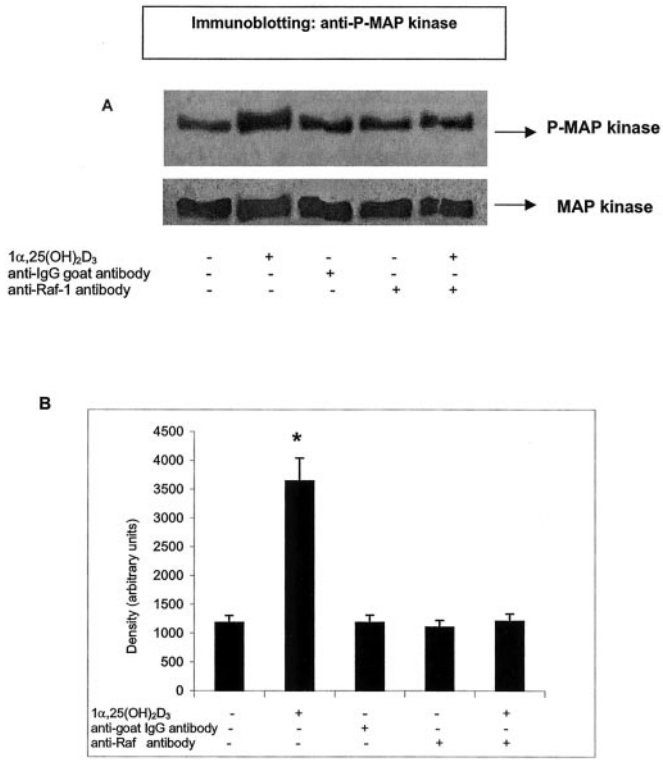


FIG. 8. **$1\alpha,25(OH)_2D_3$ stimulation of MAP kinase tyrosine phosphorylation (*P*) is suppressed by antibody inhibition of Raf-1.** After cell lysis, comparable aliquots of lysate protein (500 μg) were incubated with anti-Raf antibody (2 μg) or anti-goat IgG antibody (2 μg, as a control of the basal levels of MAP kinase) on an ice bath for 10 min. Then the lysates were exposed to $1\alpha,25(OH)_2D_3$ (1 nM) or vehicle ethanol (<0.01%) for 1 min. Proteins were resolved by SDS-PAGE followed by immunoblotting with anti-(phospho)-active MAP kinase as described under "Materials and Methods." *A*, representative immunoblot. *B*, quantification by scanning volumetric densitometry of blots from three independent experiments performed in duplicate; averages ± S.D. are given. *, $p < 0.01$ with respect to basal and $1\alpha,25(OH)_2D_3$ stimulation in the presence of anti-Raf antibody.

We finally evaluated the effects of Ras, Raf-1, and MEK inhibition on MAP kinase activity and tyrosine phosphorylation changes induced by $1\alpha,25(OH)_2D_3$. To measure MAPK activity, the enzyme from cell lysates exposed for 1 min to the hormone (1 nM) was immunoprecipitated with an anti-(phospho)-active MAPK antibody, which recognizes both the p42 and p44 active isoforms, and then incubated with [γ-^{32}P]ATP and myelin basic protein, as exogenous MAPK substrate. As previously shown (22), $1\alpha,25(OH)_2D_3$ caused a 3-fold increase in MAP kinase activity (Fig. 7). The hormone effect was abolished by either the Ras inhibitor peptide or compound PD 98059, which prevents the activation of the dual MAPK kinase MEK by Raf-1 (45). These results are in agreement with previous observations showing that PD 98059 prevents the stimulation of skeletal muscle cell proliferation by $1\alpha,25(OH)_2D_3$ (22). When muscle cell lysates were preincubated with anti-Raf-1 antibody and then exposed to $1\alpha,25(OH)_2D_3$ (1 nM, 1 min),

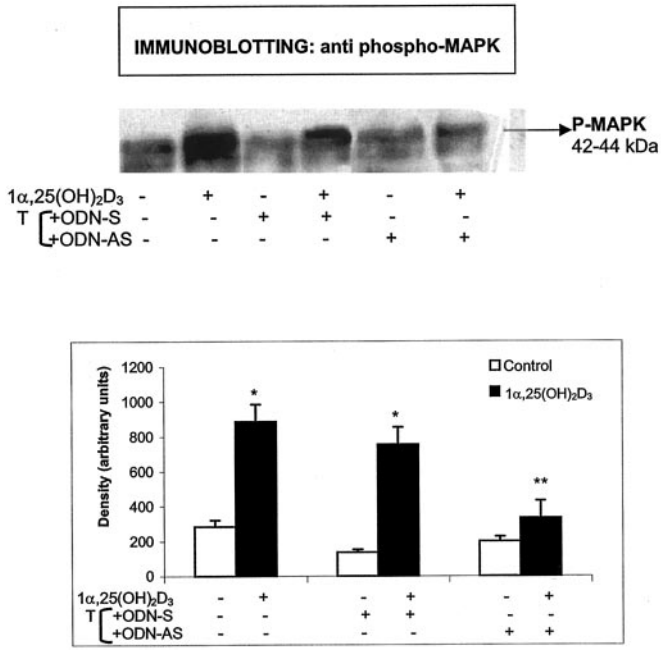


FIG. 9. **Effects of an antisense oligodeoxynucleotide against PKCα mRNA on 1α,25(OH)$_2$D$_3$-induced MAPK phosphorylation.** Muscle cells were transfected (*T*) with a sense (*ODN-S*) or an antisense (*ODN-AS*) oligodeoxynucleotide against PKCα mRNA. 48 h later the cells were exposed to 1 nM 1α,25(OH)$_2$D$_3$ for 2 min and then lysed. Proteins were resolved by SDS-PAGE followed by immunoblotting with anti-(phospho)-active MAP kinase as described under "Materials and Methods." *Top panel*, representative blot showing changes in phospho-MAPK. *Bottom panel*, quantification by scanning volumetric densitometry of the top blot from three independent experiments performed in duplicate; averages ± S.D. are given. $p < 0.01$ (*) and $p < 0.05$ (**), with respect to the corresponding control.

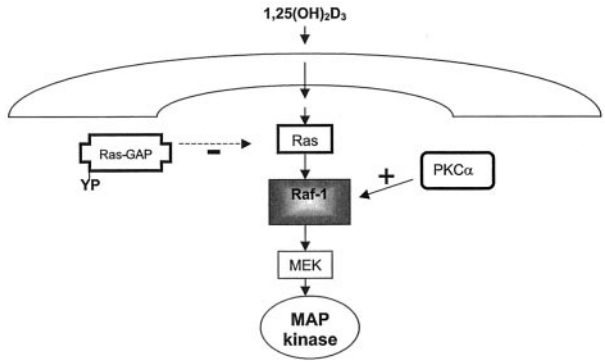


FIG. 10. **Schematic diagram indicating the chain of events leading to 1α,25(OH)$_2$D$_3$ activation of MAP kinase in skeletal muscle cells.** By acting at the plasma membrane, 1α,25(OH)$_2$D$_3$ inhibits Ras-GAP activity by tyrosine dephosphorylation (*YP*), which elicits Ras activation. The hormone also stimulates PKCα. Activated Ras along with PKCα lead to serine phosphorylation and stimulation of Raf-1 followed by MEK and MAP kinase activation.

hormone-induced MAPK tyrosine phosphorylation was also abolished (Fig. 8). Furthermore, suppression of PKCα expression in cells transfected with the antisense oligodeoxynucleotide against PKCα mRNA abolished by 70% the phosphorylation of MAPK induced by 1α,25(OH)$_2$D$_3$ (Fig. 9). These results stress the relevance of PKCα, Ras, Raf-1, and MEK in the 1α,25(OH)$_2$D$_3$-signaling pathway, which results in MAP kinase stimulation in muscle cells.

With regard to the initiation of the 1α,25(OH)$_2$D$_3$ signal that leads to activation of the MAP kinase pathway, new lines of evidence indicate that steroid hormone intracellular receptors mediate rapid, non-transcriptional stimulation of MAPK via interaction with upstream components of the cascade (46). More specifically, we recently reported that activation of MAPK by 1α,25(OH)$_2$D$_3$ in muscle cells is preceded by rapid tyrosine phosphorylation of the vitamin D receptor followed by its association with Src (47). Subsequent interaction with the Shc-Grb2-Sos-Ras complex may then occur as observed in keratinocytes (48). However, the involvement of a novel 1α,25(OH)$_2$D$_3$ membrane receptor, whose existence in various hormone target cell types has received experimental support (41, 42, 49), cannot be excluded. In summary, our results demonstrate for the first time in a 1α,25(OH)$_2$D$_3$ target cell that activation of Raf-1 via Ras (through inhibition of Ras-GAP activity by tyrosine dephosphorylation) and PKCα-dependent serine phosphorylation play a central role in hormone stimulation of the MAPK-signaling pathway (depicted in the schematic diagram of Fig. 10).

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