Activation of RAF-1 through Ras and Protein Kinase Ca Mediates 1α,25(OH)2-Vitamin D3 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 D3 (1α,25(OH)2D3) is mediated by activation of the mitogen-activated protein kinase (MAPK; ERK1/2). To understand how 1α,25(OH)2D3 up-regulates the MAPK cascade, we have investigated whether the hormone acts upstream through stimulation of Ras in 1α,25(OH)2D3-stimulated myoblasts. These effects were abolished by preincubation of cells with a specific Ras inhibitor peptide that involves Ras-GTP hydrolysis and serine phosphorylation by 1α,25(OH)2D3 (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)2D3 stimulation of Raf-1. 1α,25(OH)2D3 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)2D3 activates Ras in myoblasts. The protein kinase C (PKC) inhibitors calphostin C, bisindolylmaleimide I, and Ro 318220 blocked 1α,25(OH)2D3-induced Raf-1 serum phosphorylation, revealing that hormone stimulation of muscle cells with an antisense oligodeoxynucleotide against PKCα mRNA suppressed serine phosphorylation by 1α,25(OH)2D3. The increase in MAPK activity and tyrosine phosphorylation caused by 1α,25(OH)2D3 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)2D3 exposure with an anti-Raf-1 antibody. In conclusion, these results demonstrate for the first time in a 1α,25(OH)2D3 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 MEK1 (4), initiating a protein kinase cascade that follows with the phosphorylation and activation of the extra-cellular signal-regulated mitogen-activated protein (MAP) kinase isofoms 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 Ser-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 Ca2+-dependent classical PKCs, Ca2+-independent, novel PKCs, and Ca2+- 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

* The abbreviations used are: MEK, mitogen-activated protein (MAP) kinase (MAPK) kinase; ERK, extracellular signal-regulated kinase; PKC, protein kinase C; 1α,25(OH)2D3, 1α,25-dihydroxyvitamin D3; DMEM, Dulbecco's modified Eagle's medium; GAP, GTPase-activating protein; ODN, oligodeoxynucleotide.

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The steroid hormone 1α,25-dihydroxy vitamin D₃ (1α,25(OH)₂D₃) 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 1α,25(OH)₂D₃ stimulation of the muscle cell (myoblast) MAPK pathway revealed that PKCζ and Ca²⁺ are two upstream activators mediating the hormone effect (23). In addition, it has been shown that 1α,25(OH)₂D₃ enhancement of myoblast proliferation correlates to increased PKCa expression, whereas decreased PKCa levels are observed during the subsequent activation of muscle cell differentiation by the hormone (24). Furthermore, inhibition of PKCo expression by using antisense oligonucleotide technology resulted in a significant decrease of culture cell density and DNA synthesis, clearly showing that this isoform 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 1α,25(OH)₂D₃ 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 PKCo in Raf-1 activation.

### MATERIALS AND METHODS

#### Chemicals

1α,25(OH)₂D₃ was kindly provided by Hoffmann-La Roche. Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum, specific Ras inhibitory peptide (VPVVPPVPRRK), and protein A-Sepharose were from Sigma. Lipofectin was from Invitrogen. Sense and antisense oligodeoxynucleotides were synthesized by theDNAGency (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 (1:2000) were purchased from Santa Cruz Biotechnology and affixed to Sepharose were from Sigma. Lipofectin was from Invitrogen. Sense and antisense 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-100, and 1% Nonidet P-40) and a final wash in cold phosphate-buffered saline.

#### Antibodies

Insoluble material of lysate proteins was pelleted in a microcentrifuge at 12,000 × 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-100, and 1% Nonidet P-40) and a final wash in cold phosphate-buffered saline.

#### 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 in 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 Densitometer 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.

#### Transfection—Muscle Cells

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 and time-response studies for Lipofectin and ODNs were previously performed to establish optimum conditions for the effective blockade of PKCo expression (25). The following ODN sequences with phosphorothioate linkages throughout the entire ODN molecule were used: antisense-PKCα (AS), 5′-CTATGGTCCGGCACCACACC-3′; Y = T or C (antisense sequence against 20 nucleotides upstream of the PKCo coding region) sense PKCo: 5′-ACTTTAACGTTTGCGGGGCRATCCG-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 PKCo (human, rat, mouse, and rabbit species). Cell death in cultures under control and treatment (ODNs) conditions was measured by trypan blue staining.

#### Statistical Analysis

The 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 ± S.D. from the indicated set of experiments.
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activated 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 1α,25(OH)2D3-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)2D3 (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, 1α,25(OH)2D3 caused a time-dependent increase in Raf-1 phosphorylation in muscle cells. At the concentration used (25 nM), it is likely that sufficient amounts of the inhibitor peptide entered the cells by endocytosis to reach the 

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 1α,25(OH)2D3 on Ras-GAP tyrosine phosphorylation. As shown in Fig. 3, muscle cells treated with 1 nM

![Image](https://example.com/image1.png)

**Fig. 1.** Time course of 1α,25(OH)2D3 stimulation of Raf-1 serine phosphorylation. Chick skeletal muscle cells were incubated in the presence of 1α,25(OH)2D3 (1 nM) or vehicle (ethanol <0.01%) for the indicated times. Immunoprecipitation of Raf-1 and immunoblotting with an anti-phosphoserine 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.

**RESULTS AND DISCUSSION**

To understand how the steroid hormone 1α,25(OH)2D3 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 1α,25(OH)2D3 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-regu-

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 1α,25(OH)2D3, 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 1α,25(OH)2D3, demonstrating that Ras is necessary for 1α,25(OH)2D3-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 Kd (25 nM) for its binding to Grb2 (35).

Ras proteins play a central role in control of cell prolifera-

![Image](https://example.com/image2.png)

**Fig. 2.** 1α,25(OH)2D3-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 1α,25(OH)2D3 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 1α,25(OH)2D3 stimulation.
1α,25(OH)₂D₃ 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. This result suggests that inhibition of Ras-GTP hydrolysis is part of the mechanism by which 1α,25(OH)₂D₃ activates Ras in skeletal muscle cells.

PKC, discovered as a serine/threonine kinase (39), mediates intracellular responses to a variety of agonists including 1α,25(OH)₂D₃ (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 1α,25(OH)₂D₃ is mediated by PKC and Ca²⁺ (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 1α,25(OH)₂D₃ (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 Ca²⁺-dependent. It is well recognized that when skeletal muscle cells are subjected to 1α,25(OH)₂D₃ stimulation, a rapid increase of intracellular Ca²⁺ (22), inositol trisphosphate, and diacylglycerol.

**Fig. 3.** Transient inactivation of Ras-GAP by tyrosine dephosphorylation (P) in response to 1α,25(OH)₂D₃. Lysates from chick muscle cells exposed to 1 nM 1α,25(OH)₂D₃ 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 re-probed 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 1α,25(OH)₂D₃. Muscle cells were treated with 1 nM 1α,25(OH)₂D₃ 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 1α,25(OH)₂D₃ stimulation in the presence of PKC inhibitors.

**Fig. 5.** The PKC inhibitor Ro 318220 blocks the activation of Raf-1 induced by 1,25(OH)₂D₃. Muscle cells were treated with 1 nM 1,25(OH)₂D₃ 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 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; **, p < 0.05 with respect to 1α,25(OH)₂D₃ stimulation.
PKCα mRNA on 1α,25(OH)₂D₃-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 (LPP) to have a basal expression control. 48 h later the cells were exposed to 1 nM 1α,25(OH)₂D₃ 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.

mRNA to suppress expression of its encoded protein. Under these conditions, phosphorylation of Raf-1 induced by 1α,25(OH)₂D₃ was fully abolished (Fig. 6A, upper panel). Suppression of PKCα expression was verified by immunoblotting of cell lysates with anti-PKCα antibody (Fig. 6A, 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 1α,25(OH)₂D₃ stimulation.

We finally evaluated the effects of Ras, Raf-1, and MEK inhibition on MAP kinase activity and tyrosine phosphorylation changes induced by 1α,25(OH)₂D₃. To measure MAP kinase activity, the enzyme from cell lysates exposed for 1 min to the hormone (1 nM) was immunoprecipitated with an anti-(phosho)active MAP kinase antibody, which recognizes both the p42 and p44 active isoforms, and then incubated with [γ-³²P]ATP and myelin basic protein, as exogenous MAPK substrate. As previously shown (22), 1α,25(OH)₂D₃ 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 1α,25(OH)₂D₃ (22). When muscle cell lysates were preincubated with anti-Raf-1 antibody and then exposed to 1α,25(OH)₂D₃ (1 nM, 1 min),...
stress the relevance of PKC/H9251 along with PKC
Muscle cells were transfected (oden-S) or an antisense (ODN-AS) oligodeoxynucleotide against PKCs mRNA. 48 h later the cells were exposed to 1 nm 1α,25(OH)2D3 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 phosphoprotein. 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.

hormone-induced MAPK tyrosine phosphorylation was also abolished (Fig. 8). Furthermore, suppression of PKCa expression in cells transfected with the antisense oligodeoxynucleotide against PKCa mRNA abolished by 70% the phosphorylation of MAPK induced by 1α,25(OH)2D3 (Fig. 9). These results stress the relevance of PKCa, Ras, Raf-1, and MEK in the 1α,25(OH)2D3-signaling pathway, which results in MAP kinase stimulation in muscle cells.

With regard to the initiation of the 1α,25(OH)2D3 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).

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Fig. 9. Effects of an antisense oligodeoxynucleotide against PKCo mRNA on 1α,25(OH)2D3-induced MAPK phosphorylation. Muscle cells were transfected (oden-S) or an antisense (ODN-AS) oligodeoxynucleotide against PKCs mRNA. 48 h later the cells were exposed to 1 nM 1α,25(OH)2D3 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 phosphoprotein. 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)2D3 activation of MAP kinase in skeletal muscle cells. By acting at the plasma membrane, 1α,25(OH)2D3 inhibits Ras-GAP activity by tyrosine dephosphorylation (YP), which elicits Ras activation. The hormone also stimulates PKC. Activated Ras along with PKCα lead to serine phosphorylation and stimulation of Raf-1 followed by MEK and MAP kinase activation.
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