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Review

VDR activation of intracellular signaling pathways in skeletal muscle

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ABSTRACT

The purpose of this article is to review the activation of signal transduction pathways in skeletal muscle cells by the hormone $1\alpha,25(OH)_2$-vitamin D_3 [$1\alpha,25(OH)_2D_3$], focusing on the role of the vitamin D receptor (VDR). The hormone induces fast, non transcriptional responses, involving stimulation of the transmembrane second messenger systems adenylyl cyclase/cAMP/PKA, PLC/DAG + IP_3/PKC, Ca^{2+} messenger system and MAPK cascades. Short treatment with $1\alpha,25(OH)_2D_3$ induces reverse translocation of the VDR from the nucleus to plasma membranes. Accordingly, a complex is formed in the caveolae between the VDR and TRCP3, integral protein of capacitative Ca^{2+} entry (CCE), suggesting an association between both proteins and a functional role of the VDR in $1\alpha,25(OH)_2D_3$ activation of CCE. Stimulation of tyrosine phosphorylation cascades by $1\alpha,25(OH)_2D_3$ have demonstrated the formation of complexes between Src and the VDR. Through these mechanisms, $1\alpha,25(OH)_2D_3$ plays an important function in contractility and myogenesis.

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Contents

1. Introduction

$1\alpha,25$-dihydroxy-vitamin D_3 ($1\alpha,25(OH)_2D_3$) acts, as other steroid hormones, through two different mechanisms. In addition to regulating expression of target genes via its specific nuclear receptor (VDR), the hormone induces fast, non transcriptional responses involving stimulation of transmembrane second messenger systems, e.g. adenylyl cyclase/cAMP/PKA, PLC/DAG + IP_3/PKC, intracellular Ca^{2+} and MAPK cascades. The rapid nature and specificity by which $1\alpha,25(OH)_2D_3$ triggers the activation of these second messengers has led to the concept that interaction with a plasma membrane receptor is responsible for the initiation of its effects. However, there is controversy over its molecular characteristics. Among several proposals for non-genomic receptor identity, it has been suggested the existence of membrane associated forms of either the classical receptor (VDR) or alternatively novel hormone binding proteins. This chapter focuses on $1\alpha,25(OH)_2D_3$ regulation of intracelullar signaling pathways and its relationship to the presence of the VDR in the plasma membrane of myoblasts/myotubes, a cell system model for skeletal muscle in which $1\alpha,25(OH)_2D_3$ signal transduction mechanisms have been intensively studied. $1\alpha,25(OH)_2D_3$ is essential for normal homeostasis of intracellular calcium and growth in skeletal muscle, and thereby plays an important role in contractility and myogenesis. Consequently, a myopathy characterized by muscle

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weakness and atrophy is observed in vitamin D-deficiently states (Boland, 1986).

2. Non-genomic regulation of skeletal muscle intracellular signaling pathways by 1α,25(OH)$_2$D$_3$

2.1. Regulation of intracellular Ca^{2+} messenger system

The non-genomic regulation of intracellular Ca^{2+} in myoblasts/myotubes by 1α,25(OH)$_2$D$_3$ has been well characterized. Various aspects of this mechanism have been reproduced using differentiated soleus skeletal muscle in vitro, whereby the hormone elicits rapid effects (1–15 min) on $^{45}Ca^{2+}$ uptake, which are not blocked by inhibitors of RNA and protein synthesis but are suppressed by blockers of voltage-dependent calcium channels (VDCC) (de Boland and Boland, 1987). Interestingly, a rapid stimulation of skeletal muscle calcium fluxes in response to 1α,25(OH)$_2$D$_3$ has also been observed in vivo (Bauman et al., 1984). In myoblasts the operation of this mechanism has been firmly established by the fact that 1α,25(OH)$_2$D$_3$-dependent Ca^{2+} influx could be mimicked by the VDCC agonist BAY K8644, and plasma membrane depolarization induced by a high K^+ medium. The effects of the combined treatment with 1α,25(OH)$_2$D$_3$ and BAY K8644 or K^+ depolarization were not additive. Moreover, the action of the hormone was dependent on extracellular Ca^{2+} as they were reversible inhibited by the Ca^{2+} chelator EGTA. Furthermore, on the basis of their sensitivity to nifedipine and verapamil, the voltage-dependent Ca^{2+} channels activated in skeletal muscle cells by 1α,25(OH)$_2$D$_3$ were pharmacologically identified as from the L-type (Vazquez and de Boland, 1993; de Boland and Boland, 1994).

1α,25(OH)$_2$D$_3$ rapidly regulates muscle cell Ca^{2+} influx by guanine nucleotide binding (G) protein mediated activation of both phospholipase C and adenylyl cyclase, leading to the stimulation or PKC and PKA and subsequent activation of VDCC by phosphorylation (Sections 2.2 and 2.3, respectively).

Spectrofluorometric studies with Fura-2-loaded muscle cells have confirmed the above observations and revealed additional key information related to the regulation of muscle intracellular Ca^{2+} homeostasis by 1α,25(OH)$_2$D$_3$. The cytosolic Ca^{2+} response to the hormone involves an initial rapid sterol-induced Ca^{2+} mobilization from IP_3/thapsigargin-sensitive stores followed by cation influx from the extracellular milieu, accounting for a sustained Ca^{2+} phase which does not return to baseline as long as the cells are exposed to the sterol. This Ca^{2+} influx was shown to be contributed not only by the well established L-type VDCC mediated Ca^{2+} entry but also by a store-operated Ca^{2+} (SOC; capacitative Ca^{2+} entry, CCE) channel, therefore introducing a novel aspect into the mechanism of 1α,25(OH)$_2$D$_3$-induced Ca^{2+} influx across the plasma membrane of muscle cells (Vazquez et al., 1997a,b, 1998). The SOC influx activated by 1α,25(OH)$_2$D$_3$ was identified by being insensitive to L-type Ca^{2+} channel antagonists but was fully inhibitable by low micromolar concentrations of La^{3+} (3 μM) and Ni^{2+}. PI(polyphosphoinositide)-specific PLC blockade prior to 1α,25(OH)$_2$D$_3$ stimulation suppressed both the cytosolic Ca^{2+} transient and SOC influx. Accordingly, depletion of intracellular Ca^{2+} stores by thapsigargin reproduced 1α,25(OH)$_2$D$_3$-induced Ca^{2+} influx, inhibiting any further response to the hormone. Furthermore, 1α,25(OH)$_2$D$_3$ increased the rate of quenching of Fura-2 fluorescence by Mn^{2+}, indicating activation of Mn^{2+} influx which specifically permeates SOC channels (Vazquez et al., 1997a,b, 1998).

There is evidence on the involvement of protein kinases in the regulation of capacitative calcium influx in muscle cells by 1α,25(OH)$_2$D$_3$. It has been shown that the hormone stimulated CCE is prevented by inhibitors of PKC (calphostin C, bisindolylmaleimide) and tyrosine kinase (genistein) but unaffected by blockade of the PKA pathway (Vazquez et al., 1998). Of relevance, SOC influx stimulated by 1α,25(OH)$_2$D$_3$ is insensitive to both calmodulin (CAM) antagonists and CAM-dependent protein kinase II (CAMKII) inhibitors when added after the IP_3-mediated Ca^{2+} transient but completely abolished when added before it. Moreover, in cells microinjected with antisense oligonucleotides directed against the CAM mRNA the sterol stimulated SOC influx is reduced up to 60% respect to uninjected cells (Vazquez et al., 2000). These results suggest that the 1α,25(OH)$_2$D$_3$-induced (IP_3-mediated) cytosolic Ca^{2+} transient is required for CAM activation which in turn mediates SOC influx in a mechanism that seems to include CAMKII.

2.2. Regulation of PLC/PLD/PLA_2/PKC pathways

There is a wealth of information showing that 1α,25(OH)$_2$D$_3$ also exerts nongenomic actions at the level of transmembrane second messenger systems, which mediate the fast effects of the hormone on muscle intracellular Ca^{2+} regulation. It has been shown that 1α,25(OH)$_2$D$_3$ modifies the activity of myoblast phospholipases in a mode independent of the nucleus. Within seconds to minutes the hormone activates phospholipase C (PLC) generating the second messengers inositol trisphosphate (IP_3, a Ca^{2+} mobilizer) and diacylglycerol (DAG, a PKC activator) from membrane phosphoinositides (Morelli et al., 1993). The formation of DAG is biphasic, with the second phase independent of IP_3 release. 1α,25(OH)$_2$D$_3$ also stimulates the rapid hydrolysis of phosphatidylcholine (PC) in myoblasts by a phospholipase D (PLD)-catalyzed mechanism. PLD activity generates choline and phosphatidic acid which in turn can be converted to diacylglycerol by the action of a phosphohydrolase, accounting for the second peak of DAG observed in response to the hormone (de Boland et al., 1994). In addition, 1α,25(OH)$_2$D$_3$ activates phospholipase A_2 (PLA_2) and the subsequent release of arachidonic acid. Rapid modulation of myoblast PLC, PLD and is PLA2 is 1α,25(OH)$_2$D$_3$ specific, as 25(OH)D$_3$ and 24,25(OH)$_2$D$_3$ do not influence enzyme activities.

The rapid activation of phospholipases by 1α,25(OH)$_2$D$_3$ involves the participation of G proteins. (AlF4)- and the stable analogue GTPγS, which activate G proteins, mimic hormone stimulation of PLA2-mediated arachidonic acid (AA) release from myoblasts prelabeled with [^{3}H]AA, whereas GDPγS and Bordetella pertussis toxin pretreatment abolish 1α,25(OH)$_2$D$_3$-dependent AA release (de Boland et al., 1995). By using similar experimental approaches, it has been shown that like PLA2, hormone modulation of PLC and PLD, is mediated by a pertussis toxin-sensitive GTP-binding protein (Morelli et al., 1996).

In agreement with the 1α,25(OH)$_2$D$_3$-induced generation of DAG via PLC and PLD, it has been reported that the hormone rapidly translocates protein kinase C into the cell membrane and increases its activity in chick muscle soleus muscle in vitro as well as in cultured embryonic muscle cells. In addition, the participation of protein kinase C in the fast 1α,25(OH)$_2$D$_3$ stimulation of $^{45}Ca^{2+}$ influx through VDCC channels is supported by experimental evidence obtained with phorbol esters and DAG analogues, which mimic the action of the hormone, as well as PKC inhibitors, which reduce its effects (Massheimer and de Boland, 1992; Vazquez and de Boland, 1996). Recent studies have revealed that PKCα is the only PKC isoform activated and translocated from cytosol to the membrane upon 1α,25(OH)$_2$D$_3$ stimulation of muscle cells (Capiati et al., 2000). Moreover, transfection of specific anti-PKCα antibodies or intranuclear microinjection of antisense oligonucleotides against PKCα mRNA coupled to spectrofluorimetric analysis of changes in intracellular Ca^{2+} in Fura-2-loaded myoblasts/myotubes shows a marked reduction of hormone-dependent Ca^{2+} influx (Capiati et al., 2000, 2001).

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2.3. Regulation of the adenylyl cyclase/cAMP/PKA pathway

There are also data implying the participation of the adenylyl cyclase (AC)/cAMP pathway in the non-genomic mode of action of 1α,25(OH)$_2$D$_3$ in muscle. Physiological concentrations of the hormone elicit very fast (within 30 s) increases in AC, cAMP levels and PKA activity in both intact differentiated muscle and cultured myoblasts/myotubes. 1α,25(OH)$_2$D$_3$ stimulation of dihydropyridine-sensitive Ca^{2+} influx is abolished by specific inhibitors of AC and PKA and mimicked by forskolin and dibutyryl cAMP, involving the AC/cAMP/PKA pathway in the hormone non-genomic modulation of VDCC (Fernandez et al., 1990; Vazquez et al., 1995).

Studies with muscle cells and tissue on the effects of G protein modulators, e.g. fluoride, GTPγS, GDPγS, cholera and Bordetella pertussis toxins, on 1α,25(OH)$_2$D$_3$-mediated Ca^{2+} uptake, as well as the observation of hormone induced decrease of [^{35}S]GTPγS binding to membranes and increased ADP ribosylation of the pertussis toxin-sensitive 41 kDa substrate, led to propose that negative regulation of an inhibitory protein coupled to AC is part of the mechanism by which 1α,25(OH)$_2$D$_3$ increases Ca^{2+} influx through the cAMP dependent pathway (Vazquez et al., 1995; Boland et al., 1991). Direct evidence on the involvement of G proteins in the fast activation of adenylyl cyclase by 1α,25(OH)$_2$D$_3$ has been obtained in experiments in which the effect of the hormone on AC, GTPase and PKA activities as well as on the phosphorylation of Gαi was studied in membranes from chick skeletal muscle cells (Vazquez et al., 1997a,b). 1α,25(OH)$_2$D$_3$ stimulates AC activity provided GTP is present in the assay. High affinity GTPase, related to Gs, is unaffected by the hormone. In the absence of GTP or in the presence of a high concentration of Mn^{2+}, a condition which provides information on adenylyl cyclase activity devoid of G-protein regulation, 1α,25(OH)$_2$D$_3$ effects on AC are abolished. PKA activity is increased in cells pretreated with the hormone. Moreover, immunoprecipitation of Gαi from [^{32}P]-labeled myoblast membranes shows that 1α,25(OH)$_2$D$_3$ increases the phosphorylation of its α subunit. Therefore, these data altogether indicate that in muscle cells 1α,25(OH)$_2$D$_3$ activates adenylyl cyclase by a GTP-dependent action implying, as suggested before, amelioration of Gi function by hormone-induced αi phosphorylation (Vazquez et al., 1997a,b)

2.4. Regulation of tyrosine phosphorylation cascades

Stimulation of tyrosine phosphorylation pathways plays an important role in the mechanism which mediates the effects of 1α,25(OH)$_2$D$_3$ on muscle growth. Tyrosine phosphorylation is a crucial event in signal transduction linked to the mitogen-activated protein kinase (MAPK). Stimulation of the MAP ERK1/2 cascade may occur through activation of receptor tyrosine kinases or G protein-coupled receptors by stimulation of non-receptor Src kinases or by direct signalling to Raf via PKC. Upon phosphorylation by mitogens, ERK1/2 is translocated from the cytoplasm into the nucleus which results in the activation or induction of transcription factors leading to the expression of genes involved in the control of cellular growth (Neary, 1997).

1α,25(OH)$_2$D$_3$ rapidly promotes in cultured myoblasts tyrosine phosphorylation of ERK1/2, PLCγ and the c-myc oncoprotein (Morelli et al., 2000). Fast 1α,25(OH)$_2$D$_3$-dependent increased Src kinase activity has been observed in myoblasts (Buitrago et al., 2000).

Preincubation of muscle cells with specific Src inhibitors or their transfection with an antisense ODN against Src mRNA inhibits 1α,25(OH)$_2$D$_3$ activation of ERK1/2 involving Src as an upstream element which leads to hormone signaling through this cascade (Buitrago et al., 2001b). Of interest, recent studies applying siRNA technology, confocal immunocytochemistry and co-immunoprecipitation, have shown that 1α,25(OH)$_2$D$_3$ stimulation of Src is mediated by the sequential participation of PKCδ and protein tyrosine phosphatase (PTP)α, PTPα activating Src by dephosphorylating its Tyr530 (Buitrago and Boland, submitted).

In addition, there is evidence that calcium and protein kinase C are also involved in the stimulation of ERK1/2 by 1α,25(OH)$_2$D$_3$ (Morelli et al., 2001). Recent investigations (Buitrago et al., 2003) have established the role of PKC and besides uncovered other metabolic steps which participate in hormone upregulation of the ERK1/2 cascade. Thus, 1α,25(OH)$_2$D$_3$ causes a fast significant increase of Raf-1-serine phosphorylation, indicating activation of Raf-1 by the hormone. The PKC inhibitors calphostin C, bisindolylmaleimide I and Ro 318220 blocked 1α,25(OH)$_2$D$_3$-induced Raf-1 serine phosphorylation, revealing that PKC participates in hormone stimulation of ERK1/2 at the level of Raf-1. Moreover, application of antisense oligonucleotide technology revealed that PKCα specifically mediates this action. In addition, by using a specific Ras peptide inhibitor, Ras has also been involved in 1α,25(OH)$_2$D$_3$ activation of Raf-1. The hormone rapidly induced tyrosine dephosphorylation 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 (Buitrago et al., 2003).

Stimulation of tyrosine phosphorylation cascades by 1α,25(OH)$_2$D$_3$ through the above described mechanisms causes translocation of ERK1/2 from the cytoplasm to the nucleus in active phosphorylated form and induces the expression of the growth-related protein c-myc, as the MAPK kinase (MEK) inhibitor PD98059 abolishes stimulation of c-myc synthesis by 1α,25(OH)$_2$D$_3$ (Buitrago et al., 2001b). There is also information available on the signaling cascade leading to PLCγ activation, a relevant event which may account for the activation of PKCα (followed by that of Raf-1) via release of DAG and IP$_3$-mediated Ca^{2+}. Investigations based on the utilization of specific inhibitors and antisense technology have involved Src and phosphatidylinositol 3-kinase (PtdIns3K) in 1α,25(OH)$_2$D$_3$ stimulation of PLCγ tyrosine phosphorylation and its translocation to the cell membrane. Evidence has been obtained indicating that the hormone increases the physical association of Src and PtdIns3K with PLCα and induces a Src-dependent tyrosine phosphorylation of the p85 regulatory subunit of PtdIns3K (Buitrago et al., 2002).

There is evidence that other members of the MAP kinase superfamily are also activated by the hormone in muscle cells (Buitrago et al., 2006) as shown in different target cells (Boland et al., 2005). 1α,25(OH)$_2$D$_3$ has been shown to rapidly stimulate (within 1 min) MKK3/MKK6/p38 MAPK through Src in C2C12 cells. Of relevance, the hormone induced in the C2C12 line the stimulation of mitogen-activated protein kinase activating protein kinase 2 (MAPKAP-kinase 2) and subsequent phosphorylation of heat shock protein 27 (HSP27) in a p38 kinase activation-dependent manner. 1α,25(OH)$_2$D$_3$ also promotes the phosphorylation of JNK 1/2 (0.5–1 min). The relative contribution of ERK-1/2, p38, and JNK-1/2 and their interrelationships in hormonal regulation of muscle cell proliferation and differentiation remain to be established.

Altogether, 1α,25(OH)$_2$D$_3$ stimulates in muscle cells an intricate network of signaling components and interacting pathways, which provide a mechanism underlying regulation of muscle cellular growth by the hormone.

3. Subcellular localization and translocation of the VDR in skeletal muscle cells

The presence of the VDR in skeletal muscle has been clearly demonstrated in avian, murine and human cells and tissue by characterization of biochemical properties, detection of VDR-mRNA by RT-PCR and anti-VDR antisense ODNs, Western blot and immunocytochemical analysis using highly specific antibodies (Boland et al., 1985; Simpson et al., 1985; Costa et al., 1986; Zanello et al., 1997; Buitrago et al., 2001a; Capiati et al., 2002; Santillán et al., 2004b),

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although a recent study demonstrating the expression of small amounts of VDR mRNA in skeletal muscle, has failed to detect the receptor protein by immunohistochemistry and immunoblot assays (Wang and DeLuca, 2011). It is possible that differences in experimental conditions precluded these authors to detect considerable lower levels of VDR in muscle than in the duodenal tissue they used as positive control. Accumulation of the receptor at specific muscle cell sites like the plasma membrane/caveolae (Capiati et al., 2002; Buitrago and Boland, 2010) may be sufficient to sustain $1\alpha,25(OH)_2D_3$ triggered non-genomic signaling.

In accord with the several lines of evidence showing rapid non-genomic effects of $1\alpha,25(OH)_2D_3$ on various signaling systems, it has been observed that short treatment with the hormone induces the translocation of the VDR from the nucleus to the plasma membrane in chick skeletal muscle cells (Capiati et al., 2002). In these studies a high degree of purity of both fractions was evidenced by measurement of specific markers of subcellular components. The $1\alpha,25(OH)_2D_3$-dependent intracellular redistribution of the VDR was blocked by genistein, herbimycin or colchicine, suggesting the involvement of tyrosine kinase/s and microtubular transport in the relocation of the receptor.

In agreement with the above observations, interactions of the nuclear VDR-$1\alpha,25(OH)_2D_3$ complex with plasma membrane acceptance sites have been reported for osteoblastic ROS 17/2.8 cells (Kim et al., 1996). Plasma membrane components have been involved in the initiation of non-transcriptional rapid responses induced by $1\alpha,25(OH)_2D_3$ through activation of MAPKs in skeletal muscle cells mentioned before. Thus, there is evidence that caveolae and caveolin-1 (cav-1) participate in hormone stimulation of c-Src and MAPKs in C2C12 murine myoblasts (Buitrago and Boland, 2010). When proliferating cells were pretreated with methyl beta cyclodextrin (MβCD), a caveolae disrupting agent, under conditions in which cell morphology is not affected and no signs of apoptosis are observed, $1\alpha,25(OH)_2D_3$-dependent activation of ERK1/2, p38 MAPK and c-Src was suppressed. Similar results were obtained by siRNA technology whereby silencing of cav-1 expression abolished activation of c-Src and MAPKs induced by the hormone. Confocal immunocytochemistry and co-immunoprecipitation assays showed that cav-1 colocalizes with c-Src in the periplasma membrane zone at basal conditions, whereas $1\alpha,25(OH)_2D_3$ treatment disrupted the colocalization of these proteins and redistributed them into cytoplasm and nucleus. Moreover, confocal microscopy revealed that the hormone induces VDR translocation to the plasma membrane as shown before by cell fractionation studies (Capiati et al., 2002), and this effect is abolished by MβCD. Altogether, these data suggest that caveolae is involved upstream in c-Src-MAPKs activation by $1\alpha,25(OH)_2D_3$ and that VDR and cav-1 participate in the rapid signaling elicited by the hormone (Buitrago and Boland, 2010). In line with these observations, studies in different cell types have also demonstrated the presence of VDR associated with plasma membrane caveolae (Norman et al., 2002; Huhtakangas et al., 2004), although no evidence was presented on a functional role of the membrane VDR in non-genomic modulation of signaling pathways. Interestingly, the identification of an alternative ligand-binding pocket in the nuclear VDR has allowed to generate by computer docking a receptor conformational ensemble model providing an explanation for how VDR can have genomic and non-genomic functions (Mizwicki et al., 2004a,b, 2005).

4. Role of the VDR in the activation of skeletal muscle cell signaling by $1\alpha,25(OH)_2D_3$

In agreement with the data described above (Section 3) demonstrating translocation from the nucleus and localization of the classic VDR into the caveolae of skeletal muscle cells induced by $1\alpha,25(OH)_2D_3$, various lines of evidence indicate that membrane-associated VDR is the receptor that mediates the non-genomic effects of the hormone. Thus, the mechanism of $1\alpha,25(OH)_2D_3$ regulation of store operated Ca^{2+} influx in muscle cells supports this concept. The structural components of SOC channels are the TRPC (Transient Receptor Potential-canonical) proteins, which function as Ca^{2+} permeable channels regulated by intracellular store depletion. At present, seven mammalian TRPC proteins are at least known (TRPC1-TRPC7), which are homologs of the *Drosophila melanogaster* counterparts (Birnbaumer et al., 1996). There is molecular and immunochemical evidence which confirms the endogenous expression of a TRPC3-like protein in muscle cells. Transfection of myoblasts with anti-TRPC3 antisense ODNs shows reduced CCE induced by $1\alpha,25(OH)_2D_3$. Anti-VDR antisense ODNs also inhibit hormone-dependent SOC Ca^{2+} influx and co-immunoprecipitation of TRPC3 and VDR is observed suggesting an association between both proteins and a functional role of the receptor in $1\alpha,25(OH)_2D_3$ activation of CCE (Santillán et al., 2004a,b,c). Accordingly, it has been mentioned before (Section 3) that short treatment with $1\alpha,25(OH)_2D_3$ induces translocation of the VDR from the nucleus to plasma membranes in chick myoblasts/myotubes (Capiati et al., 2002).

TRP channels have been shown to be modulated by association of macromolecules integrating signaling supramolecular complexes. The scaffold protein INAD clusters these macromolecules through its PDZ domains (Huber et al., 1998). A functional role for an INAD-like protein in hormone activation of CCE is inferred by the reduction of $1\alpha,25(OH)_2D_3$-induced Ca^{2+} influx upon transfection of muscle cells with an anti-INAD antibody or microinjection with anti-INAD antisense ODNs (Boland, 2005). In addition to TRPC3 and VDR, other components of the putative signaling complex may be calmodulin/CaMKII and Src, which have been shown to interact with TRP proteins and the VDR (Buitrago et al., 2001a; see below), respectively, in keeping with the fact that CaM antagonists and inhibitors of CaMKII as well as of tyrosine kinases block $1\alpha,25(OH)_2D_3$-induced SOC influx (Vazquez et al., 1998, 2000).

There is evidence indicating that the VDR is involved in the non-genomic stimulation of tyrosine phosphorylation cascades by $1\alpha,25(OH)_2D_3$ in muscle cells. Coimmunoprecipitation analysis have provided evidence that $1\alpha,25(OH)_2D_3$ promotes the formation of complexes between Src and the VDR, and Src and c-myc (Buitrago et al., 2000), which can be explained by the fact that both the VDR and c-myc behave as $1\alpha,25(OH)_2D_3$-dependent tyrosine phosphorylated proteins and may interact with Src through the SH2 domain of the latter. Preincubation of myoblasts with a pool of different antisense ODNs against the VDR mRNA (AS-VDR ODNs) significantly reduces Src stimulation, further implying the VDR in hormone activation of Src (Buitrago et al., 2001a). Although not fully, ERK1/2 tyrosine phosphorylation by $1\alpha,25(OH)_2D_3$ is affected by transfection with AS-VDR ODNs implying that VDR-dependent signaling mediates hormone stimulation of the ERK cascade (Buitrago et al., 2001a; Boland et al., 2002).

5. Novel putative $1\alpha,25(OH)_2D_3$ receptors mediating non-genomic actions

First studies have suggested the existence of a $1\alpha,25(OH)_2D_3$ binding protein different from the nuclear VDR in the plasma membrane of chick enterocytes which mediates rapid hormone stimulation of intestinal Ca^{2+} transport and PKC activity (Nemere et al., 1994, 1998). Alternatively, it has been reported that annexin II may be the membrane receptor that mediates $1\alpha,25(OH)_2D_3$ -induced rapid increases in cytosolic Ca^{2+} in rat osteoblast-like cells

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ROS 24/1 which do not express the VDR (Baran et al., 2000). However, subsequent studies have provided evidence that annexin II does not bind 1α,25(OH)$_2$D$_3$ in a physiologically relevant manner casting doubts on the possibility that it is a putative membrane receptor for the hormone (Mizwicki et al., 2004a,b). It has also been reported that another cell surface receptor for 1α,25(OH)$_2$D$_3$ termed MARRS (Membrane Associated, Rapid Response Steroid binding), identical to the thiol-protein disulphide oxidoreductase Erp57/GRp58/ERp60 may mediate several 1α,25(OH)$_2$D$_3$-induced effects in various cell types, including certain cancer cells, chondrocytes and muscle (Boyan et al., 2006; Khanal and Nemere, 2007; Richard et al., 2010). In addition, recent studies have provided evidence on a novel 1α,25(OH)$_2$D$_3$ putative receptor, namely protein-disulfide isomerase-associated 3 (Pdia3), as an initiator of hormone-stimulated membrane signaling pathways (Chen et al., 2010).

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