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COMMENTARY

Nongenomic Signaling by Vitamin D

A NEW FACE OF Src

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ABSTRACT. It is well known that steroids are able to produce nongenomic effects on various cells, such as activating protein kinases, opening ionic channels, or stimulating release of second messengers. Recently, calcitriol (the hormonal form of vitamin D) has been shown to stimulate the enzymatic activity of a nonreceptor protein tyrosine kinase, Src, in keratinocytes and colonocytes. This mode of signal transduction resembles that utilized by membrane receptors devoid of intrinsic tyrosine kinase activity. There is evidence that calcitriol-activated Src plays an important role in signal transduction due to the activation of protein kinase C isozymes or a mitogen-activated protein kinase cascade. Src-mediated signaling, therefore, may be an important mediator of the physiological and pharmacological effects of calcitriol. The synthesis of vitamin D analogs capable of selective activation or inhibition of the Src-mediated signaling pathway(s) may be a new, promising approach to expanding the therapeutic scope and clinical utility of these compounds. BIOCHEM PHARMACOL **56**;10: 1273–1277, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. calcitriol; vitamin D; vitamin D receptor; nonreceptor protein tyrosine kinases; Src; signal transduction

1,25-Dihydroxyvitamin D_3 (calcitriol) is a steroid hormone capable of activating a specific nuclear receptor (VDR†). According to the traditional view, VDR and other steroid receptors bind to gene promoters, acting as transcription factors. This mode of signal transduction has been called "genomic" since activation or inhibition of gene transcription is central. The overall scheme of genomic signaling has been known for over 20 years, and its molecular mechanisms are understood in considerable detail, especially for VDR [1].

Genomic signaling has often been contrasted with the mode of signal transduction exerted by cytokines and peptide hormones. The latter agents employ specific membrane receptors and second messengers. This mode may be classified as "nongenomic" since alterations of gene transcription, although frequently encountered, are not absolutely required for the generation of a biologically meaningful signal.

For years, steroid hormones were considered incapable of nongenomic signaling, but this view is changing. Experimental evidence for nongenomic signaling has been collected for almost all types of steroid hormones, especially for vitamin D. Calcitriol has been shown to stimulate rapid formation of second messengers (ceramides, cyclic AMP, inositols, calcium) and to activate enzymes involved in signal transduction (isozymes of protein kinase C, sphingomyelinase, MAPK, Raf kinase) (for excellent reviews and editorials see Refs. [2–4]). However, the idea of nongenomic signaling in relation to steroid hormones has not been widely accepted, mainly for two reasons. First, the molecular mechanism of signal transduction has not been elucidated. It is not known whether steroid hormones use a second set of receptors for nongenomic signaling or whether classic steroid receptors are involved. Second, the physiological relevance of nongenomic signaling is still unclear.

Recent research has brought us closer to the elucidation of these questions.

NONRECEPTOR PROTEIN TYROSINE KINASES AND STEROID SIGNALING

PTKs are commonly involved in signal transduction from membrane receptors for cytokines, lymphokines, and peptide hormones [5]. Some activated receptors (e.g. receptor for epidermal growth factor) express PTK activity responsible for autophosphorylation of receptor molecules and/or phosphorylation of other downstream proteins, such as Shc (Fig. 1). Tyrosine-phosphorylated proteins attract two other signaling molecules, Grb2 and Sos (product of the *Son of sevenless* gene) (Fig. 1). Activated Sos stimulates GTP loading to Ras proteins, allowing them to transmit

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† *Abbreviations:* VDR, vitamin D receptor; MAPK, mitogen-activated protein kinase; PTK, protein tyrosine kinase; and Sos, product of the *Son of sevenless* gene.

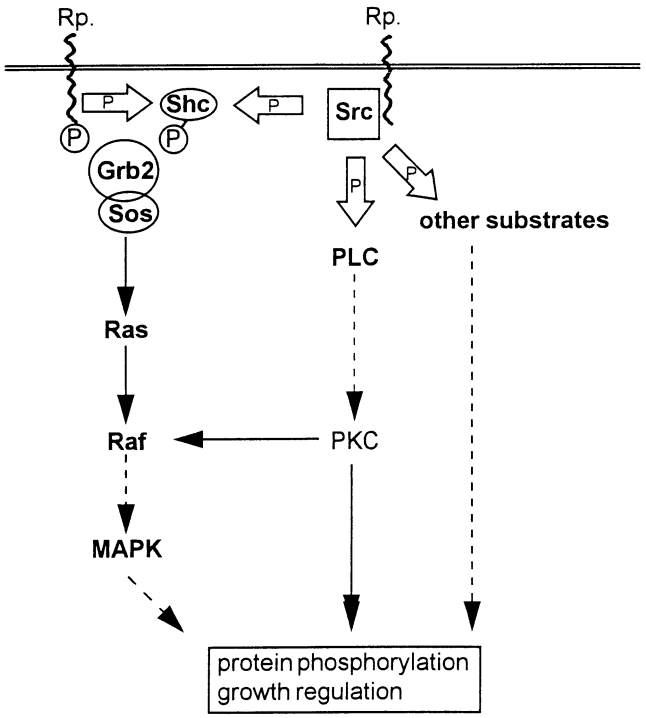

FIG. 1. Signaling pathways involving nonreceptor protein tyrosine kinases. Membrane receptors (Rp.) with intrinsic PTK activity (left) become autophosphorylated or phosphorylate Shc at tyrosine residues (Ⓟ). Receptors without PTK activity (right) recruit nonreceptor PTKs (for example Src) that phosphorylate Shc, phospholipases (PLC), or other substrates. Phosphorylated Shc activates the MAPK cascade by forming a macromolecular complex with Grb2 and Sos, stimulating GTP loading on Ras, which phosphorylates Raf and eventually MAPK. Activation of PLC leads to activation of isozymes of protein kinase C (PKC), which is also able to activate Raf independently of Raf. Tyrosine phosphorylation events are marked with ⇨. Solid arrows indicate direct effects; other effects are marked with dashed arrows. The double line symbolizes the plasma membrane.

signals to subsequent steps in the cascade, most commonly to the proteins Raf, MAPK-kinase, and MAPK [6, 7].

Recent research showed that calcitriol is able to stimulate the MAPK signaling cascade. In keratinocytes treated with calcitriol, activation of the enzymatic activities of Raf and MAPK was observed [8]. Moreover, calcitriol stimulated tyrosine phosphorylation of Shc and complex formation between Shc, Grb2, and Sos (Fig. 2). This result was unexpected because VDR does not have any intrinsic PTK activity. Therefore, another protein must have provided the PTK activity necessary to phosphorylate Shc and to trigger the signaling cascade.

Membrane receptors for some lymphokines (e.g. interleukin 2) do not demonstrate intrinsic PTK activity but are, nevertheless, able to signal via Ras. It turned out that those receptors recruit auxiliary PTKs, so-called nonreceptor PTKs, that provide the necessary kinase activity (reviewed in Ref. [5]). Nonreceptor protein kinases are grouped in eight families, the largest of which is the Src family, consisting of nine related enzymes: Src, Yes, Fyn, Lyn, Lck, Blk, Hck, Fgr, and Yrk. Those proteins associate with a variety of different receptors, such as the receptors for interleukins 2, 3, 4, 5, 6, 7, and 12, the granulocyte-macrophage colony stimulating factor, interferons, or prolactin. Remarkably, one type of receptor may associate with several different nonreceptor PTKs, increasing the diversity of lymphokine signaling.

Taking into account the ability of nonreceptor PTKs to cooperate with different, unrelated types of receptors, it was conceivable that those enzymes are also involved in steroid hormone signaling. Evidence supporting this notion has been presented very recently. Khare *et al.* [9] treated normal colonocytes in culture with calcitriol and measured Src activity in the cells. They discovered that calcitriol stimulated Src activity in a biphasic mode: the first peak of activation was seen after 1 min, followed by a second peak after 9 min. Gniadecki [10] treated normal cultured keratinocytes with calcitriol and observed an increase in Src activity after approximately 15 min (possible activation at shorter times was not investigated because of methodological limitations). In both studies, calcitriol was able to cause dephosphorylation of Src, which is an established mode of Src activation [18]. Moreover, physiologically relevant substrates for Src were identified in both studies. Khare *et al.* [9] found that activated Src associated with phospholipase C-γ, causing activation of the latter. Gniadecki [10] discovered that Src forms complexes with Shc and that Shc was tyrosine phosphorylated. Taken together, vitamin D seems to be able to activate at least two different signaling cascades via Src: (1) phospholipase C-γ → polyphosphoinositide breakdown → activation of protein kinase C isoforms and calcium mobilization; and (2) Shc phosphorylation → activation of Grb2 and Sos → activation of Ras → activation of MAPK (Fig. 2).

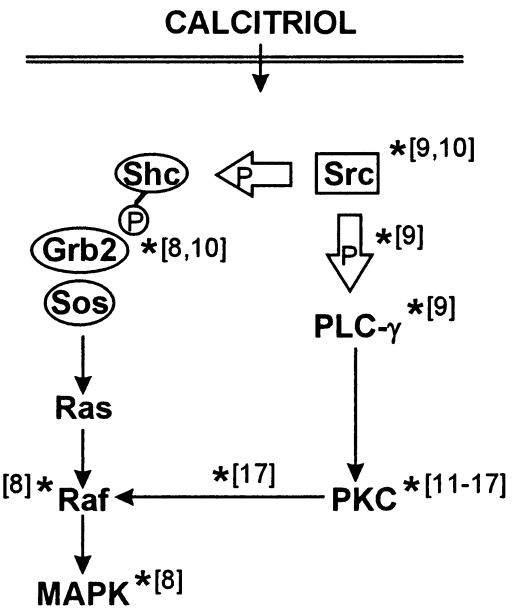

FIG. 2. Experimental evidence for nongenomic vitamin D signaling. Processes verified experimentally are marked with asterisks; other connections are hypothetical. Numbers in brackets refer to articles cited in the reference list.

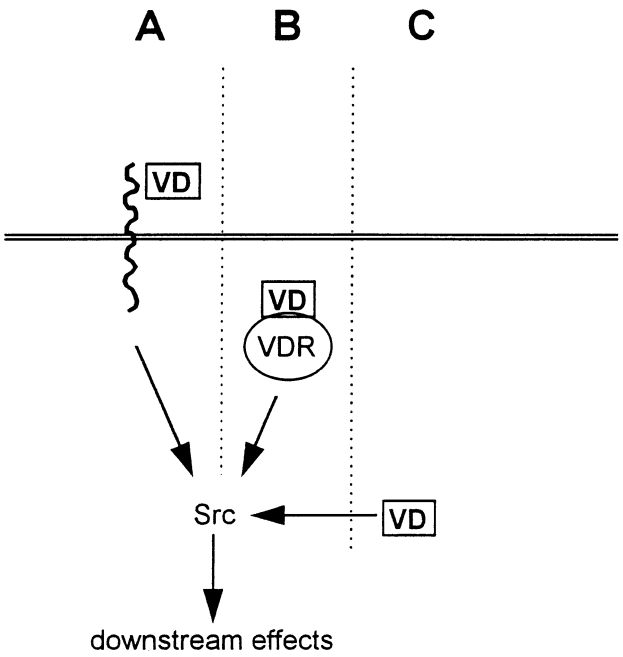

FIG. 3. Molecular mechanisms of nongenomic signaling. Three alternative possibilities are presented. (A) vitamin D (VD) binds to a specific, yet unidentified membrane receptor; (B) nongenomic effects are mediated by activated VDR; and (C) vitamin D is able to directly activate signaling proteins, such as Src. It is conceivable that activation of nonreceptor PTKs is a central event in nongenomic signaling.

The discovery of the involvement of Src in calcitriol signaling in two different cell types brings us closer to the understanding of nongenomic vitamin D signaling. However, the mechanisms leading to Src activation remain elusive. Is another, membrane-bound form of vitamin D receptor involved, as postulated by Nemere, Norman and colleagues some time ago [19, 20] (Fig. 3)? There is not much evidence for that possibility thus far. VDR seems to be the only high-affinity cellular receptor for calcitriol. Membrane events do not seem to be necessary for Src activation, as shown by Khare *et al.* [9], who employed calcitriol encapsulated in liposomes. Calcitriol provided in this form was able to activate Src despite the fact that liposomes deliver the hormone directly to the cytoplasm, bypassing the cell membrane. These data make the involvement of a membrane receptor unlikely.

Is it possible that calcitriol activates Src via VDR? This is surely the simplest scheme, implying that one receptor is involved in both genomic and nongenomic signaling (Fig. 3). Although experimental evidence supporting this scenario is lacking, some recent data indicate that VDR may be directly involved in nongenomic signaling [8]. In immunoprecipitation experiments, VDR molecules coprecipitate with phosphorylated Shc in calcitriol-treated keratinocytes. Thus, Shc and VDR form a macromolecular complex, due to either a direct binding between proteins or via an as yet unidentified linker protein(s) (Fig. 3). The role of VDR in this complex (functional or bystander) remains to be identified.

PHYSIOLOGICAL SIGNIFICANCE OF NONGENOMIC VITAMIN D SIGNALING

As stated above, the significance of nongenomic signaling, including the effects of Src activation, is largely unknown. Most researchers were content with the biochemical evidence of nongenomic events and did not explore their relevance for cell physiology. There is some evidence that tyrosine phosphorylation events (initiated possibly by Src activation) play an important role in the modulation of cellular growth by calcitriol. The effects of vitamin D on cell proliferation have been studied thoroughly in keratinocytes. In this cell type, calcitriol is able to both stimulate and inhibit cell proliferation, depending on hormone concentration and cell culture conditions [21, 22]. In conditions favoring growth stimulation, calcitriol activates the Raf → MAPK signaling cascade, which is probably triggered by the activation of Src and Shc (Fig. 2). Inhibition of Raf by antisense oligonucleotides abrogates the stimulatory effects of calcitriol on keratinocyte growth, strongly suggesting that activation of Raf and MAPK is an important mechanism of growth regulation by vitamin D [8].

In addition to growth regulation, calcitriol is a potent stimulator of keratinocyte differentiation. Very interestingly, tyrosine phosphorylation events and Src activation have been shown to be important, if not indispensable, for epidermal cell differentiation [23, 24]. For example, treatment of keratinocytes with high concentrations of calcium and calcium ionophores induces Src activation and cell differentiation [23]. Thus, it is tempting to speculate that calcitriol-mediated Src activation is an important signaling pathway leading to cell differentiation.

It is also relevant to study whether vitamin D is able to activate/suppress other nonreceptor PTKs. Such a finding would not be surprising in view of the fact that membrane receptors associate promiscuously with many different nonreceptor PTKs, e.g. the erythropoietin receptor is able to recruit members from the Src, Jak, and Syk families. Activation of different nonreceptor PTKs by vitamin D may have physiological consequences. For example, the protein Yes, a member of the Src family, is dephosphorylated and inactivated during keratinocyte differentiation, which is precisely the opposite phenomenon to that seen for Src [23]. Thus, activation of Yes, rather than Src, by vitamin D could conceivably result in the maintenance of the undifferentiated state. As discussed below, elucidation of these pathways is important, in view of the therapeutic application of vitamin D compounds in the treatment of cancer and hyperproliferative skin disorders such as psoriasis.

ADVANTAGES OF NONGENOMIC SIGNALING

In the case of membrane-anchored receptors, the activation of auxiliary kinases and the generation of second messengers are necessary to carry the message from the membrane

to target organelles, such as the nucleus. Because VDR is able to travel to the nucleus and activate/suppress appropriate genes, nongenomic signaling seems to be superfluous at first glance. However, genomic signaling lacks two important properties: (1) speed (it takes several hours to change the transcription level of a gene, translate the required protein in sufficient amounts, and introduce post-translatory modifications; this time is even longer when multiple proteins are involved in signal transduction); and (2) amplification and fine tuning of the signal (the response is proportional to the number of occupied VDRs and lasts as long as activated VDRs are bound to gene promoters). Therefore, nongenomic signaling may be viewed as a useful complementary mechanism to genomic mechanisms, further increasing the diversity and improving the regulation of the effects of steroid hormones.

CONCLUSIONS AND PERSPECTIVES

Evidence accumulated hitherto indicates unequivocally that steroid hormones, including vitamin D, are capable of nongenomic signaling, in a manner similar to the agents utilizing membrane receptors. A new aspect of nongenomic signaling is the involvement of nonreceptor protein kinases, particularly Src, in the early stages of steroid signaling. Despite this progress, research on nongenomic signaling by steroids is still in its infancy. The major breakthrough would be to describe the molecular mechanisms of Src activation and resolve the question as to whether new putative receptors or classic steroid receptors mediate nongenomic signaling.

Recently, calcitriol and its analogs have been introduced in the therapy of psoriasis, where they inhibit excessive epidermal cell proliferation and promote cell differentiation [25]. Much effort has been put into synthesizing compounds with limited potency to induce hypercalcemia but that have retained high antiproliferative activity. A new approach may be the use of analogs that preferentially activate either the genomic or the nongenomic responses. Some evidence suggests that the synthesis of such compounds is feasible [26, 27]. In view of the involvement of Src in keratinocyte growth and differentiation, vitamin D compounds modulating the activity of this kinase may be particularly interesting candidates for psoriasis treatment.

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