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Review

Steroid signal transduction activated at the cell membrane: from plants to animals★

Ewa Marcinkowska[1]✉ and Antoni Więdłocha[2]

[1]*Department of Reproductive Immunology, Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wrocław, Poland; and* [2]*Department of Biochemistry, Institute for Cancer Research, The Norwegian Radium Hospital, N-0310 Oslo, Norway*

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Steroid hormones in plants and in animals are very important for physiological and developmental regulation. In animals steroid hormones are recognized by nuclear receptors, which transcriptionally regulate specific target genes following binding of the ligand. In addition, numerous rapid effects generated by steroids appear to be mediated by a mechanism not depending on the activation of nuclear receptors. Although the existence of separate membrane receptors was postulated many years ago and hundreds of reports supporting this hypothesis have been published, no animal membrane steroid receptor has been cloned to date. Meanwhile, a plant steroid receptor from *Arabidopsis thaliana* has been identified and cloned. It is a transmembrane protein which specifically recognizes plant steroids (brassinosteroids) at the cell surface and has a serine/threonine protein kinase activity. It seems that plants have no intracellular steroid receptors, since there are no genes homologous to the family of animal nuclear steroid receptors in the genome of *A. thaliana*.

Since the reason of the rapid responses to steroid hormones in animal cells still remains obscure we show in this article two possible explanations of this phenomenon. Using 1,25-dihydroxyvitamin D_3 as an example of animal steroid hormone, we review results of our and of other groups concordant with the hypothesis of membrane ste-

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✉Corresponding author: Ewa Marcinkowska, Department of Reproductive Immunology, Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, R. Weigla 12, 53-114 Wrocław, Poland; e-mail: marcinko@iitd.pan.wroc.pl webpage http://www.ii.uni.wroc.pl/~ema/

Abbreviations: BR, brassinosteroids; BRI-1, brassinosteroid-insensitive 1; ER, estrogen receptor; FGF-1, fibroblast growth factor-1; PI 3-K, phosphatidylinositol 3-kinase; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; RE, response element; nVDR, nuclear vitamin D receptor.

roid receptors. We also review the results of experiments performed with ovarian hormones, that led their authors to the hypothesis explaining rapid steroid actions without distinct membrane steroid receptors. Finally, examples of polypeptide growth factor that similarly to steroids exhibit a dual mode of action, activating not only cell surface receptors, but also intracellular targets, are discussed.

STEROID HORMONES IN ANIMALS AND PLANTS

All plant and animal steroid hormones are cholesterol derivatives, synthesized by the organisms. Physiologic effects of steroid hormones are necessary for regulation of growth, development and homeostasis. The majority of steroid hormones retain the four ring core of cholesterol (Fig. 1) with one exception of vitamin D, where B-ring is broken. In vertebrates steroids are a very large group of hormones which includes sex hormones, glucocorticoids, corticosteroids, metabolites of vitamin D_3 and neurosteroids. In insects ecdysteroids are absolutely necessary for development, growth and molting (Bollenbacher *et al.*, 1975). Also in plants steroid hormones (brassinosteroids) are essential for growth and differentiation. Mutant plants deficient in brassinosteroid synthesis exhibit dwarfism, delayed senscence, reduced fertility and light-independent development (Wang & Chory, 2000).

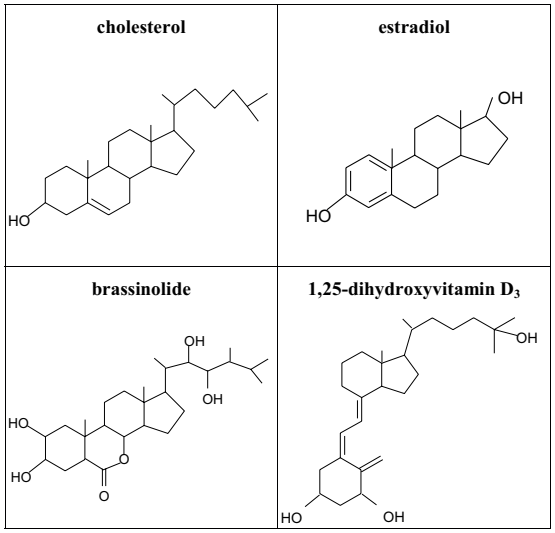

Figure 1. Chemical structures of cholesterol and some steroid hormones.

Brassinolide (the most active brassinosteroid from plants), estradiol (an animal ovarian hormone) and 1,25-dihydroxyvitamin D_3 (the hormonally active form of vitamin D).

NUCLEAR STEROID RECEPTORS IN ANIMALS

The nuclear steroid hormone receptor superfamily has been known for over two decades (Evans, 1988). This superfamily includes also receptors for retinoids, thyroid hormones and a subfamily of so called orphan receptors with still unidentified ligands. All these receptors act as ligand inducible transcription factors. They share many structural similarities, all have a short DNA-binding domain with two characteristic zinc fingers. After binding of the ligand to the hydrophobic ligand binding domain, the activated receptor recognizes appropriate response element (RE) in the promoter region of the target gene. For their transcriptional activity the majority of nuclear receptors need to dimerize forming either homodimers (e.g. estrogen receptor) or heterodimers (e.g. vitamin D receptor/retinoic acid receptor). Although studies on the molecular mechanism of transcriptional regulation by nuclear receptors are progressing very rapidly, the understanding of the process is still far from satisfactory (Di Croce *et al.*, 1999). Greatly simplified, the whole process could be described as follows. Binding of the activated nuclear receptor to the appropriate RE induces a bend in the promoter DNA. Changes in conformation of the nuclear receptor allow coactivator proteins to interact with the complex. Since some components of the coactivator complex exhibit histone acetyltransferase activity, formation of this com-

plex results in acetylation of histones and remodelling of the DNA structure. This in turn allows the transcriptional machinery to reach the target gene. In some cases binding of the liganded receptor to the response element leads to decreased transcription of the target gene in a not yet fully understood way. Activation of a nuclear receptor by its ligand induces cellular effects after a relatively long time, ranging from dozens of minutes to several hours.

RAPID RESPONSES TO STEROID HORMONES

Most steroid hormones induce in the target cells responses too rapid to involve transcriptional regulation. The classic example was published by Hans Selye in 1941 (Selye, 1941) reporting immediate anesthesia after administration of progesterone at pharmacologic concentrations. Hundreds of publications documenting rapid effects of steroids have been published since then. These effects resemble cellular responses induced by growth factors and protein hormones that are recognized at the cell membrane by specific transmembrane protein receptors. Different steroid hormones induce in their target cells rapid raises of intracellular calcium levels (Machelon *et al.*, 1998). They are able to activate protein kinases C and A (Kelly *et al.*, 1999), G proteins (Rosner *et al.*, 1999), MAPK cascade (Marcinkowska *et al.*, 1997) or phospholipase A_2 (Schwartz *et al.*, 1988). Since these studies have been performed on different experimental models, and cellular responses to various steroid hormones are also different it is very difficult to give a summary of membrane-mediated responses generated by steroid hormones. This is why only a selected group of papers will be discussed here in order to present two possible explanations for the rapid cellular responses to steroid hormones.

NON-TRANSCRIPTIONAL CONTRIBUTION TO ACTIVATION OF PROLIFERATION BY OVARIAN HORMONES

Ovarian hormones are potent stimulators of mitogenesis for breast cancer cells. Experimental model of hormone-induced mitogenesis of breast cancer cells served a group of researchers from Naples to show the biological meaning of rapid cellular responses generated by these hormones. In a series of exhaustive papers the authors showed that estradiol induces proliferation through activation of the Src/Ras/Erks signalling pathway in MCF-7 and T47D cells (Migliaccio *et al.*, 1993; 1996; Castoria *et al.*, 1999) similarly to progestins that activate this same pathway in T47D cells (Migliaccio *et al.*, 1998). Rapid activation of this pathway was necessary for S-phase entry of the cells and could be blocked by anti-estrogens as well as by anti-progestins. The authors have proved that the expression of both estrogen nuclear receptors (ER) and progesterone nuclear receptors (PR) in breast cancer cells is necessary for the activation of Src/Ras/Erks. They showed that liganded ER interacts with c-Src, while PR interacts with ER and these interactions create a cross-talk between the transcriptional and non-transcriptional activities of ovarian hormones. Recently the same authors found that also the phosphatidylinositol (PI) 3-kinase/Akt signal transduction pathway mediates the estradiol-induced S-phase entry and induction of cyclin D1 in MCF-7 cells (Castoria *et al.*, 2001). Hormone-induced stimulation of PI 3-kinase and Akt is Src dependent. Pull-down experiments revealed that estradiol triggers association of ER with Src and PI 3-kinase, which does not occur in the absence of the hormone.

Summarizing, the authors of the cited papers do not postulate the existence of separate membrane receptors responsible for the rapid cellular responses to ovarian hormones. On the basis of the experimental data they hy-

pothesize that rapid estradiol- or progestin-induced activation of cell signalling results from different scaffolding of proteins belonging to the signal transduction pathways. This hypothesis does not violate the widely accepted paradigm that nuclear receptors are the only steroid receptors in animal cells. It should be noticed, however, that specific binding sites for estrogens in the cell membrane of endometrial cells were described many years ago (Pietras & Szego, 1977) and that some reports suggest that this membrane estrogen binding protein is derived from the same transcript as nuclear ER (Razandi *et al.*, 1999). It has been shown recently that ER associate with calveolin, an important structural component of calveolae (Razandi *et al.*, 2002). Calveolae are cell membrane microstructures, where various intracellular signals are initiated.

METABOLITES AND ANALOGS OF VITAMIN D AS TOOLS FOR STUDYING THE NON-TRANSCRIPTIONAL MECHANISM OF ACTION OF STEROID HORMONES

Vitamin D differs from other steroid hormones in its chemical structure (see Fig. 1). It is a derivative of cholesterol as all other steroid hormones, but its B ring of the cholesterol core is broken. This feature makes the molecule extremely flexible. The physiological functions of vitamin D are very broad (Jones *et al.*, 1998). Only its hydroxylated metabolites are active in the organism. The most active is 1,25-dihydroxyvitamin D_3, but also 24,25-dihydroxyvitamin D_3 is necessary for bone formation. Initially, emphasis was placed only on the function of vitamin D metabolites in skeleton development (so called "calcemic function"). However, further studies revealed its role in differentiation of many cell types. This twofold activity of vitamin D metabolites raised hope that chemical modifications of the molecule would enable uncoupling of the calcemic and differentiation-inducing activities of the hormone. In fact there are hundreds of vitamin D analogs described in the literature at present, and many of them fulfill these requirements. Some of them are deprived of their calcemic function, but retain the ability to induce cell differentiation (Bouillon *et al.*, 1995). Although the nuclear vitamin D receptor (nVDR) was cloned many years ago, its crystal structure had not been solved until recently (Rochel *et al.*, 2000). It was believed that analogs of vitamin D that are particularly active in the induction of cell differentiation cause conformational changes in the structure of nVDR responsible for their higher biological activity. Surprisingly, the crystal structures of nVDR complexed to 20-epi analogs (superagonists) of vitamin D revealed that the lipid-binding domain conformation is the same as for 1,25-dihydroxyvitamin D_3 (Tocchini-Valentini *et al.*, 2001). So there must be another mechanism through which this higher differentiation-inducing activity could be achieved.

The phenomenon that the affinity of a particular vitamin D analog for nVDR is not directly proportional to its differentiation-inducing activity had been observed before the solution of the nVDR crystal structure was obtained. For example (see Table 1): an analog of vitamin D_3 with extended side chain (analog 1), synthesized in the Pharmaceutical Research Institute in Warsaw (Poland), which binds nVDR with a 10 times higher affinity than 1,25-dihydroxyvitamin D_3, induces cell differentiation similarly to the parent compound (Chodyński *et al.*, 1997; Marcinkowska *et al.*, 1998). Another analog with unsaturated side-chain (analog 2), also synthesized in the Pharmaceutical Research Institute in Warsaw (Chodyński *et al.*, 2002), is able to induce HL-60 cell differentiation much stronger than 1,25-dihydroxyvitamin D_3 (Marcinkowska, 1998). In the same HL-60 cells 1,25-dihydroxyvitamin D_3 induces rapid activation of Erk kinases (Marcinkowska *et al.*, 1997). This response is too fast to be regulated trans-

Table 1. Differentiation of HL-60 cells induced by 1,25-dihydroxyvitamin D_3 and by its two analogs.

The table uses data taken from Marcinkowska *et al*. (1998) (analog 1) and Marcinkowska (1998) analog 2). HL-60 cells after 96-h treatment with the compounds were tested for their ability to:
– phagocyte *Saccharomyces cervisiae*. Mean percentage of phagocyting cells is presented.
– reduce yellow NBT[1] to dark blue formazan. Mean percent of positive cells is presented.
– express the CD11b and CD14 cell surface markers. Table shows mean channel of fluorescence (MC) of cells expressing either CD11b or CD14 measured in flow cytometry.
Means ± S.D. were calculated from at least three experiments.

Compound	Conc. [nM]	Phagocytosis [%]	NBT reduction [%]	CD11b [MC]	CD14 [MC]
Control	none	0.7 ± 0.8	2.6 ± 2.8	7 ± 1	6 ± 2
$1,25\text{-}(OH)_2\ D_3$	1000	89.2 ± 0.8	94.8 ± 2.5	51 ± 2	91 ± 27
	10	54.3 ± 3.3	64.5 ± 5.8	38 ± 13	94 ± 23
	0.1	ns	ns	22 ± 5	48 ± 0.1
Analog 1	1000	87.7 ± 3.6	93.2 ± 1.3	54 ± 22	68 ± 26
	10	49.3 ± 13.2	50.8 ± 5.5	39 ± 18	108 ± 32
	0.1	ns	ns	10 ± 3	14 ± 2
Analog 2	1000	73.8 ± 3.8	86.3 ± 5.2	59 ± 16	104 ± 17
	10	67.8 ± 7.9	77.6 ± 6.9	55 ± 10	112 ± 18
	0.1	46.5 ± 12.4	59.4 ± 14.3	46 ± 8	117 ± 34

[1]NBT, nitro blue tetrazolium; ns, not screened

criptionaly *via* activation of nVDR. Erks are not only activated in response to 1,25-dihydroxyvitamin D_3, but also are translocated to the cell nucleus (Fig. 2). Also analog 2 with strong differentiation-inducing properties (Table 1) is able to induce rapid activation of Erks in HL-60 cells (Marcinkowska & Kutner, 2002).

An elegant evidence that a mechanism unrelated to nVDR is involved in cell differentiation induced by an analog of vitamin D was published recently (Ji *et al*., 2002). The analog of vitamin D described there is 40 times less calcemic than 1,25-dihydroxyvitamin D_3, but retains similar cell differentiation-inducing activity. It was shown that this analog up-regulates nVDR expression less than 1,25-dihydroxyvitamin D_3. When an oligonucleotide containing the binding sequence for nVDR was added to the cells, the differentiation response to the analog was less inhibited than the differentiation in response to 1,25-dihydroxyvitamin D_3.

MEMBRANE VITAMIN D RECEPTOR PROTEIN

The contribution of a specific membrane receptor to the generation of rapid cellular responses to vitamin D was postulated already in the past (Nemere & Szego, 1981a; 1981b; Lieberherr *et al*., 1989). A candidate for such a membrane receptor, a 65-kDa protein, was purified from basal-lateral membranes of chick epithelial cells by ion-exchange chromatography (Nemere *et al*., 1994), but cDNA for this protein has not been cloned so far. However, biochemical data and immunochemical data obtained using a specific antibody (Ab99) revealed that this protein is not related to nVDR. This protein has been found not only in the membranes of cells from chick intestine, but also in cells from chick kidney and brain (Jia & Nemere, 1999; Nemere *et al*., 2000; Nemere & Campbell, 2000). And the most important: a protein recognized by the same Ab99 antibody was found in rat

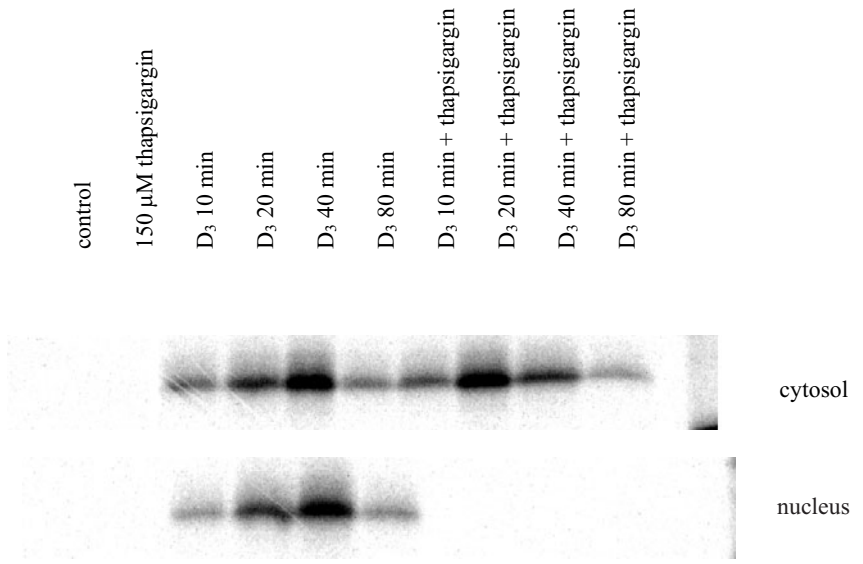

Figure 2. Activation and nuclear translocation of extracellular signal-regulated kinases (Erks) in HL-60 cells in response to 1,25-dihydroxyvitamin D_3.

The figure is prepared according to Marcinkowska *et al*. (1997). Serum-starved HL-60 cells were treated with 1 μM 1,25-dihydroxyvitamin D_3 for indicated times. Then the cells were lysed and Erks were immunoprecipitated separately from the cytosolic and nuclear fractions. Activation of Erks was assayed by incorporation of γ-^{32}P into myelin basic protein. Autoradiogram obtained in a representative experiment is presented. Thapsigargin (150 μM), which in an indirect manner blocks translocation of proteins to the cell nucleus, was used as a control of appropriate separation of cellular fractions (Greber & Gerace, 1995; Sweitzer & Hanover, 1996).

chondrocytes. The rapid responses to 1,25-dihydroxyvitamin D_3 in these cells could be blocked by the antibody (Nemere *et al.*, 1998; Pedrozo *et al.*, 1999; Schwartz *et al.*, 2002).

Another candidate for the membrane vitamin D receptor is annexin II (Baran *et al.*, 2000). In rat osteoblastlike cells (ROS 24/1) [^{14}C]1,25-dihydroxyvitamin D_3 bromoacetate binds specifically to annexin II, and anti-annexin II antibodies inhibit vitamin D-induced increase in intracellular calcium. It is not clear if in other cell types annexin II serves as a membrane vitamin D receptor. For example in rat matrix vesicles derived from rat chondrocytes it does not, since anti-annexin II antibodies do not inhibit the rapid responses generated by 1,25-dihydroxyvitamin D_3 (Schwartz *et al.*, 2002).

RECEPTORS FOR PLANT STEROID HORMONES

Brassinosteroids (BRs) are critical for the regulation of plant growth and development (Clouse & Sasse, 1998). In order to find a receptor or other crucial components of BR signal transduction pathways mutants of *A. thaliana* insensitive to BRs and incapable of being rescued by exogenous BR application were screened (Clouse *et al.*, 1996). Mutations were located in the *brassinosteroid-insensitive1* (*BRI1*) gene which encodes a transmembrane receptor kinase (Li & Chory, 1997). The receptor consists of an extracellular domain with 25 tandem leucine-rich repeats that resemble the repeats found in animal hormone receptors, a transmembrane 70 amino-acid domain

and a cytoplasmic kinase domain with serine/threonine specificity (Friedrichsen *et al.*, 2000; Oh *et al.*, 2000). It has been shown that BRs are direct ligands of BRI1 receptors (Wang *et al.*, 2001). Upon activation by the ligand BRI1 receptor becomes autophosphorylated (Oh *et al.*, 2000). Downstream signalling events have not been recognized as yet, however, negative-feedback regulation by a kinase-associated protein phosphatase has been suggested (Rodriguez, 1998). It seems that BR signalling through membrane receptors is widespread in the plant kingdom, since a rice homolog of BRI1 was found to mediate BR sensitivity as well (Yamamuro *et al.*, 2000).

POSSIBLE BIOLOGICAL FUNCTION OF MEMBRANE STEROID RECEPTORS

It is believed that steroid hormones enter animal cells due to their lipophilic nature by simple diffusion, and that this entry is regulated by extracellular concentration resulting from the balance between synthesis and degradation of the hormone. However, the possibility that cellular entry of steroid hormones is mediated by appropriate membrane receptors should be taken into consideration (Nemere & Szego, 1981a). Actually some data supporting this hypothesis have been provided (Nemere *et al.*, 2000). Intestinal epithelial cells untreated and treated with 1,25-dihydroxyvitamin D_3 were stained with an anti-membrane VDR antibody (Ab99) and investigated by electron microscopy. The experiments revealed that in the hormone-treated cells the protein recognized by this antibody is translocated to the cell nucleus after 10 s. The presence of cell-surface receptors, as it appears to be in the case of 1,25-dihydroxyvitamin D_3, may represent a new mechanism for membrane crossing. Active transport or at least facilitated diffusion of the hormone through the membrane might be an advantage, particularly at a lower extracellular level of the vitamin. Additionally, receptor-mediated transport of steroids into cells might be advantageous for the regulation by the cellular homeostasis-maintaining system compared to free diffusion.

It must be mentioned here that at least some polypeptide growth factors, in addition to their classic cell-membrane-mediated activity, like steroid hormones, exhibit also an intracellular mode of action similar to that of steroid hormones (Mason, 1994). The high molecular mass isoforms of FGF-2 and nuclear FGF-3 are the best described examples of endogenous nuclear growth factors. Furthermore there is an increasing body of evidence that some of the polypeptides are able to enter cells as exogenous proteins (Więdłocha, 1999; Clague & Urbe, 2001). Although the mechanism of cell entry is poorly understood, the intracellular location of exogenous growth factors indicates that they can act in different cellular compartments and likely exhibit distinct actions.

It has been shown that FGF-1 binding to a high affinity tyrosine kinase receptor is necessary for cell entry (Klingenberg *et al.*, 1998). After receptor binding followed by endocytosis, the growth factor is able to traverse cellular membranes in order to reach the cytosol and the nuclear compartment. Inside the cells FGF-1 binds to a 42 kDa protein named FIBP (FGF-1 binding protein) which localizes mainly in the cell nucleus and partly in the cytosol (Kolpakowa *et al.*, 1998). It does not quite fit into the paradigm that protein growth factors act only through surface receptors inducing signaling by phosphorylation of intracellular second messengers. However, this dual mechanism of FGF-1 signaling seems to be required to induce cell proliferation at least in some types of cells (Więdłocha *et al.*, 1996; Klingenberg *et al.*, 1998).

Vitamin D_3 as well as other steroid hormones also exhibit a dual mode of action. Intracellularly, by direct binding to nVDR and by a putative (not yet cloned) specific cell-sur-

face receptor regulating a novel-signaling pathway. It is interesting that although steroid hormones and growth factors represent chemically and structurally different agents, they are able to activate similar second messengers like PKC, PKA, PLC, MAP kinases or increase the intracellular level of Ca^{2+} in a rapid, transcription-independent manner regulated by membrane associated receptors. This phenomenon indicates that certain part of the signaling mechanism is common for many different regulatory agents.

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