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STEROID HORMONE INTERACTIONS WITH TARGET CELLS: CROSS TALK BETWEEN MEMBRANE AND NUCLEAR PATHWAYS*

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Running Title: Membrane and Nuclear Actions of Steroid Hormones

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List of Nonstandard Abbreviations:

(ER) estrogen receptors, **(TR)** thyroid hormone receptors, **(VDR)** vitamin D receptors, **(nER)** nuclear ER, **(ERE)** estrogen response element, **(KO)** knockout, **(SRCs)** steroid

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receptor coactivators, **(CBP)** CREB binding protein, **(P/CAF)** CREB binding protein associated factor, **(SERMs)** selective estrogen receptor modulators, **(LBD)** ligand binding domain, **(mERs)** membrane ERs, **(SRFC)** steroid receptor fast-action complex, **(PI3K)** phosphatidylinositol 3-phosphate kinase, **(PKC)** protein kinase C, **(EGF)** epidermal growth factor, **(EGFR)** EGF receptor, **(GnRH)** gonadotropin-releasing hormone, **(ANGELS)** activators of nongenomic estrogen-like signaling, **(TH)** thyroid hormone, **(TR)** TH receptor, **(RXR)** retinoic acid receptor, **(T_3)** 3,5,3'-triiodo-L-thyronine, **(TRE)** TH response element, **(SMRT)** silencing mediator of TH and retinoid action, **(NCoR)** nuclear co-repressor, **(T_4)** L-thyroxine, **(DBDS)** DNA-binding domains, **(mTRs)** membrane TRs, **(SR)** sarcoplasmic reticulum, **(APD)** action potential duration, **(PKM_2)** Pyruvate kinase monomer, **(STAT)** signal transducer and activator of transcription, **(MEK)** MAPK kinase, **(VDRE)** vitamin D response elements, **(VDRKO)** nVDR knockout, **(mVDR)** membrane receptors for $1,25(OH)_2D_3$, **(1,25D_3-MARRS)** Membrane-Associated Rapid Response to Steroids, **(PTH)** parathyroid hormone, **(TGFβ)** transforming growth factor β, **(PKA)** protein kinase A, **(MISS)** membrane-initiated steroid signaling, **(NISS)** nuclear-initiated steroid signaling., **(MAPK)** mitogen activated protein kinase, **(CaMK)** CA^{2+}/calmodulin-dependent protein kinase, **(eNOS)** endothelial nitric oxide synthase, **$[Ca^{2+}]_i$** intracellular calcium concentration.

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ABSTRACT

The biological effects of steroid hormones are mediated by receptors associated with the plasma membrane as well as located inside of target cells. This perspective focuses on recent advances in our understanding of the integration that occurs between membrane-associated rapid signaling events and various changes in gene transcription that modulate the function and phenotype of steroid responsive cells. Three frequently studied members of the steroid hormone receptor superfamily, the estrogen receptors (ERs), the thyroid hormone receptors (TRs), and the vitamin D receptors (VDRs), are included to illustrate the emerging concepts. Each of these hormones has been conclusively shown to function at multiple subcellular sites leading to a continuum of signals intimately linked by intracellular crosstalk. Understanding the molecular mechanisms by which these steroid hormones and their receptors transduce cellular signals, will allow us to create new pharmacologic therapies aimed at treatment of a variety of human diseases affecting the cardiovascular system, the reproductive system, the skeletal system, the nervous system, the mammary gland, and many others.

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Introduction

We have entered a new era in understanding the mechanisms by which steroid hormones exert their effects on target cells. This era is characterized by increased appreciation for the interacting network of responses that begin immediately upon exposure of cells to steroid and culminate in changes in gene expression affecting function and phenotype. Understanding this continuum of change, including the potential for crosstalk with other pathways and the elaborate feedback mechanisms that are activated, provides unprecedented opportunity to develop novel therapeutics that direct specific responses of target cells. As a scientific community, we have moved past the arguments concerning whether membrane, cytoplasmic or nuclear receptor-mediated pathways take precedence, and on to an era in which we are challenged to understand the complexities of steroid hormones as key integrators of cellular function.

Confusion persists in the literature, much of which has accumulated from inconsistent vocabulary usage in scientific publications. In this perspective, we use terminology defined below. ***Genomic*** refers to any action of a hormone that leads to a change in gene transcription, regardless of whether the classical nuclear receptor for that steroid hormone is involved. ***Nongenomic*** is used for changes that occur in pre-synthesized cellular machinery that occur independently of new messenger RNA transcription. This term should not be used when referring to rapid changes that clearly initiate new transcription. ***Nuclear receptor-mediated*** refers to changes that require direct or indirect actions of the classical nuclear steroid hormone receptors. ***Post-transcriptional*** defines changes requiring new protein translation or to ribosomal activation without new gene transcription. ***Post-translational*** protein modifications

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include changes in glycosylation state, lipid addition, and phosphorylation. ***Rapid*** refers to any action that takes place on a time scale from milliseconds to minutes. ***Long term*** includes effects that occur many hours or even days after hormone addition. ***Intermediate*** includes those events occurring within a few hours and not necessarily persisting for a day, comprising many of the actions involved in crosstalk between genomic and non-genomic responses. ***Membrane-initiated*** is limited to use when the effect of the steroid clearly involves changes in membrane protein activity such as occur with surface receptors, ion channels, or membrane-associated signaling complexes. This term also is appropriately used when referring to changes in membrane lipids such as changes in fluidity or to lipid hydrolysis.

This perspective emphasizes steroid effects on signaling pathways, new mechanisms of crosstalk in target cells, and potential and required receptors that may represent pharmacological targets. We chose three diverse steroid hormone receptor family members, estrogen receptors (ER), thyroid hormone receptors (TR), and vitamin D receptors (VDR), to explain the emerging concepts, but note that these concepts apply to other steroid hormone receptor superfamily members.

Estrogen (E_2)

Classical ERs. The classical mode of action of E_2 involves binding to the well-characterized nuclear ER (nER) which functions as a transcription factor regulating expression of a variety of functionally diverse genes. Target genes modulated by E_2 include those encoding matrix and structural proteins, regulatory enzymes, surface receptors, ion channels and transcription factors. Microarray and cluster analyses

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demonstrate clearly that treatment of cells with E_2 changes gene expression including genes directly responsive to E_2 through the nER and also genes lacking a clearly defined estrogen response element (ERE) in the promoter. Interestingly, in a microarray study using ovariectomized mice treated for 6 hrs with E_2, the vast majority of genes that reproducibly changed were not altered in parallel experiments using ER-α deficient mice (Watanabe et al., 2002). This indicates that both the rapid and long-term effects of E_2 require the nER.

Two classical nERs exist and are termed ER-α and ER-β. Three different ER knockout (KO) mouse models have been developed including those carrying a null mutation in ER-α only, ER-β only, or both genes (αβERKO). Mice with deletions of both receptors have a phenotype similar to those lacking ER-α only, but display an ovary-specific pathology that involves the ER-β (Hewitt and Korach, 2003). Like other nuclear receptors, nERs function as ligand-dependent transcription factors that require coactivators to manifest their stimulatory and inhibitory transcriptional effects. Numerous coactivators have been identified including steroid receptor coactivators (SRCs) and the cointegrators CBP and P/CAF. A recently proposed model presents a nER activated either by E_2 binding or by a cell membrane-initiated phosphorylation-dependent signaling pathway. In this model, the activated nER then recruits selected members of the multifunctional coactivator and cointegrator families which then by modulating histone acetylase activity alter chromatin structure and hence transcriptional efficiency (Ratajczak, 2001).

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Selective Estrogen Receptor Modulators (SERMs). Modulators of ER function collectively have been termed SERMs and fall into several subclasses including the anti-estrogens and the newly described selective estrogen receptor subtype modulators (SERSMs) (Meegan and Lloyd, 2003). This class of molecules is the subject of keen interest because the ligand binding domain (LBD) of the nER provides a molecular target for rational drug design. As the appreciation of the mechanism of action of E_2 evolves to include membrane, cytoplasmic, and nuclear events, the potential for development of new compounds targeting the function of the ER at specific cellular sites increases, thus providing an exciting opportunity for development of novel tissue specific drugs targeting a variety of human diseases.

Non-classical Actions of ER, Membrane ERs (mERs). While it is appreciated that nERs translocate between the cytoplasm and the nucleus, it is apparent that E_2 also binds to receptors that translocate to the plasma membrane (Pedram et al., 2002). Debate continues over whether these mERs are completely indistinguishable from nERs except by cellular localization (Singh et al., 2002), whether they represent novel ER receptors (Toran-Allerand et al., 2002) or whether structural changes target them to a separate pool localizing to the membrane. Recent reports concerning the structure of mERs remain conflicting, and provide evidence for alternatively spliced variants of ER-α (Figtree et al., 2003), amino acid substitutions (Razandi et al., 2003), and fatty acylation (Li et al., 2003) as explanations for membrane targeting of the ER-α. The mER frequently localizes to plasma membrane subdomains rich in caveolin, especially in endothelial cells where this has been well studied (Zhu and Smart, 2003). In cells in which caveolin levels are low or

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absent, such as neurons, the mER can associate with other specialized membrane subdomains. This discovery opens the door to understanding the role of steroid hormone regulated plasma membrane signaling complexes (signalosomes) comprised of receptors, signaling molecules, second messengers, and scaffolding proteins that together facilitate rapid target cell responses.

Signal Transduction Pathway Effects; Pathway Crosstalk. The biological effects of E_2 originate from activation of specific signaling pathways that integrate the membrane and nuclear actions of the hormone. Treatment of target cells with E_2 activates several different kinase cascades that originate at the cell surface, most likely in caveolae where mERs are concentrated. The existence of this signaling complex is demonstrated in endothelial cell plasma membranes, where isolated caveolae contain mER-α coupled to $G_{\alpha i}$ capable of activating eNOS. The complex is termed steroid receptor fast-action complex, or SRFC (Chambliss and Shaul, 2002). Downstream of activation of the SRFC by E_2, tyrosine kinase-MAPK and Akt/protein kinase B signaling are activated, which along with stimulation of heat shock protein 90 binding to eNOS and changes in Ca^{+2} homeostasis lead to eNOS phosphorylation and calmodulin-mediated stimulation (Chambliss and Shaul, 2002). Microarray analysis of gene expression in vascular endothelial cells treated with E_2 for 40 min showed that some 250 genes were up-regulated and that this could be prevented by inclusion of an inhibitor of phosphatidylinositol 3-phosphate kinase (PI3K/Akt), LY294002 (Pedram et al., 2002). Interestingly, Li et al (2003) showed using human endothelial cells that the alternately spliced variant of mER that they call ER46 more efficiently modulated membrane-

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initiated estrogen actions leading to eNOS activation than did the full-length mER-α that they call ER66. Very recently, it was demonstrated that low physiologically relevant concentrations of E_2 increase the intracellular pH of rat aortic smooth muscle cells through a mechanism involving inositol 1,4,5-triphosphate, protein kinase C (PKC), and MAPK (S. Incerpi, personal communication). Together, these findings emphasize the importance of interacting signaling and transcriptional actions controlling some of the unique vasoactive properties of E_2 (Mendelsohn, 2002).

E_2 stimulates growth of a variety of cells including cancer cells. Like many polypeptide growth factors, E_2 can stimulate the Src/ras/erk pathway in epithelial derived cancers such as those from mammary and prostate. E_2 treatment of mammary-derived MCF-7 cells triggers association of ER with Src and p85, the regulatory subunit of PI3K leading to DNA synthesis (Migliaccio et al., 2002). Using this cell line, it was shown that treatment with epidermal growth factor (EGF) phosphorylated serine and tyrosine residues in ER-α and that a direct interaction of ER-α and EGF receptor (EGFR) resulted (Marquez et al., 2001). Complex formation then stimulated cell proliferation and prevented apoptosis independent of the ERE-dependent transcriptional activity of ER-α.

In the nervous system, it is clear that E_2 influences many aspects of neural function that affect cognition, behavior, stress responses, and reproduction. Many of these effects are transcription-independent and involve activation of cell surface receptors and ion channels. E_2 causes hyperpolarization of hypothalamic gonadotropin-releasing hormone (GnRH) neurons and simultaneously regulates coupling of G protein receptors to potassium channels in dopaminergic neurons (Kelly et al., 2002). It has been proposed that gonadal steroid regulation of gonadotropin release may be almost completely

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attributed to a transcription-independent mechanism (Wiebe, 1997) involving activation of ion channels, Ca^{2+} mobilization and associated signaling pathways. Longer-term genomic actions are required for the transcriptional events governing replacement of secreted proteins.

In the skeleton, a new paradigm has arisen for explaining the actions of E_2 on bone resorption, an issue intricately linked to our understanding of postmenopausal osteoporosis. The discovery of ANGELS, a class of molecule involved in a novel extranuclear ER signaling activity in bone cells, has redefined the field (Moggs et al., 2003). In this model, activation of the mER-α in the caveolae of bone-forming osteoblasts transmits survival signals through activation of Src/Shc/erk pathway and prolongs the lifetime of the osteoblast (Manolagas et al., 2002). At the same time, E_2 delivers a pro-apoptotic signal to bone resorbing osteoclasts, shortening their lifetime. Numerous studies support the idea that effects of E_2 on gene expression in bone cells trigger crosstalk between signaling pathways and transcriptional events that maintain proper bone mass.

Thyroid Hormone (TH)

Classical TR. The classical mechanism of action of TH depends upon the presence of the nuclear TH receptor (TR)β1, a member of the nuclear superfamily of hormone receptors (Yen, 2001). TRβ1 forms homodimers or heterodimers with other nuclear hormone receptors, such as retinoic acid receptor (RXR), nuclear VDR and nER. When associated with its natural ligand, 3,5,3'-triiodo-L-thyronine (T_3), heterodimeric TR sheds co-repressor proteins and recruits co-activators, forming a transactivator complex

that binds to the positive upstream TH response element (TRE) of TH-responsive genes activating gene expression (Yen, 2001). Unliganded heterodimeric TR represses positive TREs. The dissociation of co-repressors that accompanies binding of T_3 by TR defines de-repression of the TRE and the recruitment of co-activators to the TR complex defines activation. TR also interacts with certain negative TREs.

Among co-activators for TR are p300, SRC-1 and Trip230 (Yen, 2001). The principal co-repressors are silencing mediator of TH and retinoid action (SMRT) and nuclear co-repressor (NCoR). TR was once thought to be a nuclear protein, rather than a mobile component of cytoplasm that translocates when complexed with its hormone ligand. Several laboratories, however, have recently established that there is a pool of extranuclear TR that translocates within minutes to the nucleus when intact cells are exposed to T_3 (Zhu et al., 1998) or to L-thyroxine (T_4) (Davis et al., 2000), a TH analogue that, when deiodinated, yields T_3. This translocation represents a transcription-independent effect on protein trafficking (see below) and does not require T_3 binding to TR; thus, the process of genomic hormone action is not analogous to the nuclear uptake of the steroid hormone-steroid receptor complex from cytoplasm. Because nuclear actions of TH require gene transcription and translation, the biological endpoint of such actions is seen hours after exposure of thyroprival cells to TH.

Microarray studies have revealed a broad spectrum of genes positively modulated and, to a lesser extent, negatively modulated, by TH (Miller et al, 2001). This is not surprising in light of the numerous reports of susceptibility of individual genes to regulation by TH. In these microarray studies, a distinction between primary and secondary regulatory effects of TH has not been clear.

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The DNA-binding domains (DBDs) of the nuclear superfamily of receptor proteins are located near the N-terminus and share structural homologies. At the C-terminal end of the DBD in TR is a hinge region that is a principal site of interactions of co-repressors and co-activators with the receptor (Yen, 2001). The C-terminal ligand-binding domain (LBD) of TR is a site of spontaneous mutations that lead to clinical resistance to TH action. TR-dependent actions of TH are significant because of the interfaces between transcription-dependent and independent mechanisms that have been reported (Davis and Davis, 2002)

Non-classical actions of TH action, Membrane TRs (mTRs). Several actions of TH do not require intranuclear liganding of T_3 by TR and are thus nonclassical actions (Davis and Davis, 2002). Usually reported in cells deprived of TH, then acutely exposed to T_4 or T_3, these effects are rapid in onset, i.e., seconds to minutes and can be reproduced in the presence of translational inhibitors or in the absence of the nTR. The latter models include CV-1 cells that lack TRβ1. These actions occur at the plasma membrane--largely in terms of ion channel and ion pump activities, at the level of certain cytoplasmic proteins, at the ribosome and Golgi apparatus, and upon the cytoskeleton. Interestingly, TH affects signal transduction pathways particularly, the MAPK; ERK1/2 cascade (Lin et al., 1999). TH also affects mitochondrial respiration directly and by more complex effects involving the mitochondrial genome or truncated forms of certain nucleoproteins, including RXR, that are imported into mitochondria (Wrutniak-Cabello C, 2002).

The actions of TH on membrane ion handling were recently reviewed (Davis and Davis, 2002) and include prolonged opening of the Na^+ channel, increased plasma membrane and sarcoplasmic reticulum (SR) Ca^{2+}-ATPase activity, increased inward

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rectifying K^+ current and decreased action potential duration (APD) when the untreated hypothyroid heart and TH-exposed hypothyroid heart are compared. APD is a complex phenomenon to which several ion currents may contribute. Acute effects of TH on myocardial contractility occur in humans (Schmidt et al., 2002), suggesting that certain of the actions at the cellular level, e.g., on SR Ca^{2+}-ATPase activity (Davis and Davis, 2002) to increase SR Ca^{2+} content or perhaps on reverse-mode Na^+-Ca^{2+} exchange when the Na^+ channel is affected, may have clinical consequences. Acute effects of TH on myocardial contractility also have been documented in animal models.

Plasma membrane Ca^{2+}-ATPase effects occur in non-heart cells including L-6 mouse myoblasts, in which increased activity of the Na^+/H^+ antiporter occurs in response to T_3 (Incerpi et al., 1999). This effect could contribute to the ability of cells to recover from acid loads.

Other Effects of TH. Pyruvate kinase monomer (PKM_2) binds T_3 preventing PK tetramer formation and activation of the kinase. Interactions of TH with other cytosolic proteins can regulate nuclear uptake of T_3, but, in contrast to steroid hormone action, are not prerequisites to nuclear effects. Specific mRNAs may be stabilized and translation rates may be affected by TH (Davis and Davis, 2002).

TH regulates the conversion of soluble actin to fibrous actin (F-actin) in astrocytes (Siegrist-Kaiser et al., 1990). T_4 and 3,3',5'-triiodo-L-thyronine (reverse T_3) are effective in this model of hormone action and T_3 is inactive. Among the manifestations of this effect is internalization of the cell surface deiodinase that converts T_4 to T_3 and modulation of laminin-astrocyte integrin interaction.

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T_4 in physiologic concentrations promotes nuclear uptake of cytoplasmic TR (Davis et al., 2000), of signal transducer and activator of transcription (STAT) proteins (Davis and Davis, 2002) activated MAPK (Lin et al., 1999) and of p53 (Shih et al., 2001). These effects originate from the cell surface and can be reproduced when entry of T_4 into the cell is prevented. T_3 in physiological concentrations does not promote nuclear translocation, but does cause transport of Trip230, a co-activator protein for TR, from the Golgi apparatus to the cell nucleus. Nuclear uptake of proteins promoted by TH analogues is blocked by inhibition of MAPK signal transduction.

Signal Transduction Pathway Effects; Pathway Crosstalk. T_4 rapidly activates the MAPK cascade in a variety of cells (Lin et al., 1999). This effect is initiated at the cell surface, requires phospholipase C and PKC activation and depends on participation of Ras, Raf-1, MAPK kinase (MEK). The downstream consequences of hormonal activation of MAPK include serine phosphorylation of several nucleoproteins, including STAT1α and STAT3 (Davis and Davis, 2002) TRβ1 (Lin et al., 2003) ERα (S. Zhang, F.B. Davis, P.J. Davis: unpublished observations) and p53 (Shih et al., 2001). The activated MAPK docking site on TR has been identified (Lin et al., 2003) and is conserved in other nuclear steroid hormone receptors. Each of these specific phosphorylation steps alters the transactivator functions of STAT proteins, TR, ER and p53. Functions include STAT-dependent growth factor and cytokine effects, the shedding of co-repressor proteins by TR and recruitment of co-activators, and ER-mediated effects on cell proliferation. Crosstalk between the STAT and MAPK pathways is clearly a transcription-independent action of TH (Davis and Davis, 2002), as is

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crosstalk between E_2 and TH signal transduction. Interestingly, *genomic* effects of ER and TR involve interactions between these two members of the nuclear superfamily of hormone receptors (Vasudevan et al., 2002).

Actions of iodothyronines on MAPK and PKC isoforms may be important to plasma membrane ion transport. For example, the stimulation of the Na^+/H^+ exchanger by T_3 is blocked by PD 98059 (S. Incerpi: personal communication), an inhibitor of the MAPK cascade. Plasma membrane Ca^{2+}-ATPase activity is modulated by PKC, and T_4 activates PKC and the calmodulin-dependent calcium pump (Davis and Davis, 2002). Promotion of nuclear uptake of TR by the action of T_4 at the cell membrane also is blocked by treatment of cells with PD 98059.

A body of work (reviewed in Davis and Davis, 2002) indicated that TH can rapidly increase $[Ca^{2+}]_i$. Incerpi et al. (personal communication) recently confirmed that TH acutely increases $[Ca^{2+}]_i$ in thyroprival L-6 myoblasts by a MAPK-dependent mechanism and that intracellular Ca^{2+} stores are the source of increased $[Ca^{2+}]$. During chronic exposure, we propose that TH is a factor that contributes to the level of cellular $[Ca^{2+}]_i$ in nonexcitable cells or resting $[Ca^{2+}]_i$ in excitable cells.

Enhanceosomes and nonclassical hormone action. Immunoprecipitation of activated MAPK in nuclear fractions from T_4-treated cells yield a complex of proteins including TRβ1, RXR, STAT1α and p53. Missing in hormone-exposed cells are TR co-repressor proteins SMRT and NCoR; present in the complex are co-activators. This complex of nucleoproteins resembles the enhanceosome of transactivators and proteins that modify transactivator effects (Carey, 1998). The enhanceosome is thought to help

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uncoil tightly DNA in nucleosomes. The enhanceosome-like complex we described in TH-exposed cells may be a vehicle by which nucleoproteins are organized and which also may facilitate transcription when T_3 is bound by the complex.

Nature of the cell surface receptor for TH. Many actions of TH occur in plasma membrane preparations and in intact cells that do not express TR. Kinetics of hormone-binding have been described in plasma membrane preparations (Davis and Davis, 2002) that contain Ca^{2+}-ATPase; the structure-activity relationships of TH analogues in these kinetic studies do not mimic those of the hormone with TR. Thus at the cell surface there exist receptors for TH that are distinct from the nuclear receptor. The cell surface receptors for iodothyronines have not been isolated or cloned. Analyzed in the context of their mediation of MAPK pathway stimulation, they appear to be G protein-coupled receptors and may be identified by their ability to bind tetrac (Davis and Davis, 2002). Tetrac is not an agonist, but blocks the action of bioactive TH at the receptor site.

Interfaces between genomic and nongenomic actions of the TH. The distinction between genomic and nongenomic actions of TH becomes blurred when MAPK-dependent downstream effects of TH in the cell nucleus are studied. As noted above, TH-activated MAPK serine phosphorylates several nuclear transactivator proteins. This posttranslational modification alters the transcriptional activity of the transactivators, such as nuclear hormone superfamily members, STAT proteins or p53. TH can separately affect a gene and activity of the gene product. For example, sarcolemmal Ca^{2+}-ATPase activity is enhanced acutely by TH, particularly, T_4, and the transcription of

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Ca^{2+}-ATPase genes also is modulated by T_3. Disparate roles for T_4 and T_3 in, respectively, transcription-independent and dependent functions represent a convenient concept, but these roles are not preserved in other cell functions regulated by TH.

1,25-Dihydroxyvitamin D_3 ($1,25(OH)_2D_3$

Classical VDR (nVDR). Like nER and nTR, the nVDR functions as a dimeric transcriptional regulator acting on vitamin D response elements (VDRE) and whose function is tightly regulated by co-activators and co-repressors (MacDonald et al., 2001). Conflicting data exists as to whether the nVDR functions as part of the plasma membrane signalosome. Using nVDR knockout (VDRKO) mice, it was shown that the loss of nVDR did not abrogate the nongenomic increases in $[Ca^{2+}]_i$ or the activation of PKC in primary osteoblasts (Wali et al., 2003). In contrast, (Erben et al., 2002) reported that deletion of the DBD of the nVDR eliminated both genomic and nongenomic functions of $1,25(OH)_2D_3$. The nVDR is a highly mobile receptor (Barsony and Prufer, 2002) whose presence at multiple cellular sites is clear. One of the more interesting developments of the nVDR is its role in cell growth and tumorigenesis. Using cells from both wild type and VDRKO mice, it was shown that both normal and transformed mammary cells require nVDR for $1,25(OH)_2D_3$-mediated growth inhibition to occur (Welsh et al., 2002). Similar dependence on nVDR was reported for other epithelial-derived cell types growth inhibited by $1,25(OH)_2D_3$ including prostate, colon, and skin.

Non-classical actions of VDR, Membrane VDR (mVDR)/1,25-MARRS. The non-classical actions of $1,25(OH)_2D_3$ were recently reviewed (Farach-Carson and

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Nemere, 2003). There is every reason to suspect that membrane receptors for $1,25(OH)_2D_3$ (called mVDR or VDR_{mem}) interact with scaffolding proteins, move to lipid rafts or other plasma membrane subdomains, and form multi-protein signaling complexes. Indeed the composition of $1,25(OH)_2D_3$-responsive matrix vesicles blebbed off from skeletal cells is clearly distinct from the plasma membrane (Boyan et al., 2002). $1,25(OH)_2D_3$ treatment of osteoblasts rapidly triggers sphingomyelin turnover (Liu et al., 2000), a phenomenon known to be associated with lipid rafts. Signaling by $1,25(OH)_2D_3$ also is clearly associated with Src (Gniadecki, 1998), another common component of lipid rafts. Controversy abounds over whether the nVDR is an essential component of the membrane signalosomes. While analog specificity, responsiveness in nVDR free membrane preparations and matrix vesicles, and some studies with knockout mice argue for separate receptors, other observations hint at novel roles for nVDR at the plasma membrane. The discovery of $1,25D_3$-MARRS (Membrane-Associated Rapid Response to Steroids), itself a $1,25(OH)_2D_3$ binding entity with multiple predicted regulatory phosphorylation sites (Nemere and Farach-Carson, submitted), indicates that there are likely to be multiple receptors for $1,25(OH)_2D_3$, as with TH. The next few years should bring resolution to some of these controversies as the molecular identity and function of the candidate receptors are critically examined and tested in several laboratories.

Analogs and Antagonists of $1,25(OH)_2D_3$. Hundreds of synthetic analogs of $1,25(OH)_2D_3$ have been synthesized, and their therapeutic potential well reviewed (Brown, 2001). Convincing evidence has accumulated that conformationally flexible ligands of the VDRs define their binding specificity by their ability to interact with the

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LBDs of target receptors. The concepts that emerge from numerous studies with various natural metabolites and synthetic analogs that act as agonist or antagonists of the VDRs are (1) that the orientation and rigidity of the side chain is critically important, and (2) that the position of the A ring in relation to the C/D rings as determined by rotation around the 6,7 single carbon bond in the seco B ring is a major determinant (Norman et al., 2001). Interestingly, it was recently proposed that the receptor specific actions of $1,25(OH)_2D_3$ could be considered as parallels to the SERMs, and that the current collection of $1,25(OH)_2D_3$ agonist and analogs might be used clinically as selective receptor modulators of the vitamin D endocrine system (Pike et al., 2002).

Signal Transduction Pathway Effects; Crosstalk. Like E_2 and TH, $1,25(OH)_2D_3$ interacts with peptide hormones that in this case include parathyroid hormone (PTH), transforming growth factor β (TGFβ), and inflammatory cytokines to modulate cellular responses. Changes in gene transcription occur in response to $1,25(OH)_2D_3$ alone and as a result of crosstalk between $1,25(OH)_2D_3$-activated pathways and peptide hormone-activated pathways. For example, $1,25(OH)_2D_3$ triggers a rapid phosphorylation of serine residues on IκBα in monocytes, which synergize with PKC dependent signaling pathways to regulate NFκB translocation and signaling (Berry et al., 2002). Likewise, Smad proteins transduce signals downstream of TGFβ that mediate crosstalk between TGFβ and $1,25(OH)_2D_3$ signaling in osteoblasts (Gurlek and Kumar, 2001). PTH treatment of osteoblasts activates protein kinase A (PKA), which in turn modulates calcium channel function, and in the presence of $1,25(OH)_2D_3$-activated CaMK alters $[Ca^{2+}]_i$, and regulates secretion of osteoclastic coupling factors (Bergh, Duncan and

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Farach-Carson, submitted). In muscle cells, phospholipase C redistribution and activation occurs as a consequence of rapid 1,25$(OH)_2D_3$-induced signal transduction involving c-Src and PI3K (Buitrago et al., 2002). These are but a few of many recent examples of rapid effects on signal transduction induced by 1,25$(OH)_2D_3$, all of which interact with other pathways to regulate cell fate and function, including those regulated by the nVDR.

Microarray analysis reveals that treatment of cells with 1,25$(OH)_2D_3$ alters gene expression, only some of which are mediated directly through binding of nVDR complexes to VDRE in target genes. Microarray screening of cells treated with 1,25$(OH)_2D_3$ for various time periods produces data on changes that can be clustered into distinct groups. A recent analysis in our laboratory produced 23 gene clusters involving several hundred genes (Farach-Carson and Xu, 2002). In addition to those predicted to be activated directly by the classic nVDR acting on the VDREs of target genes such as osteopontin, numerous changes in gene expression were seen at three hrs including those encoding matrix proteins, signaling molecules, stress response proteins, cell cycle regulators, and transcription factors having no VDRE in their promoters. The advent of array technology provides a boon to the study of rapid actions of 1,25$(OH)_2D_3$ especially those genomic actions not mediated directly through the nVDR. Such phenomena might have been lumped erroneously into the broad category of rapid/nongenomic effects of 1,25$(OH)_2D_3$ in the not so distant past, when the truth is that they involve transcriptional effects <u>independent</u> of the nVDR. This explains why inhibitors of transcription or translation can block some of the rapid effects even though they have a pharmacological profile consistent with the mVDR rather than the nVDR.

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General Concepts

In summary, there is an increasing body of literature documenting the integrated function of the rapid, intermediate and long-term effects of steroid hormones in modulating target cell behavior through interacting transcriptional events and signaling pathways. These effects were the subject of a recent summer conference (Nemere et al., 2003) where the terminology MISS (membrane-initiated steroid signaling) and NISS (nuclear-initiated steroid signaling) was proposed. The field is moving quickly, with many new observations since this subject was reviewed (Falkenstein et al., 2000). Figure 1 presents a model for receptor signaling and crosstalk that provides a template for understanding the complex actions of steroid hormones that play a vital role in human health and disease. While the details of the molecular mechanisms are unique to each hormone, the overarching theme that is rapidly unveiling clearly points to many commonalities in the integrated function of the members of the steroid hormone family.

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Footnotes.

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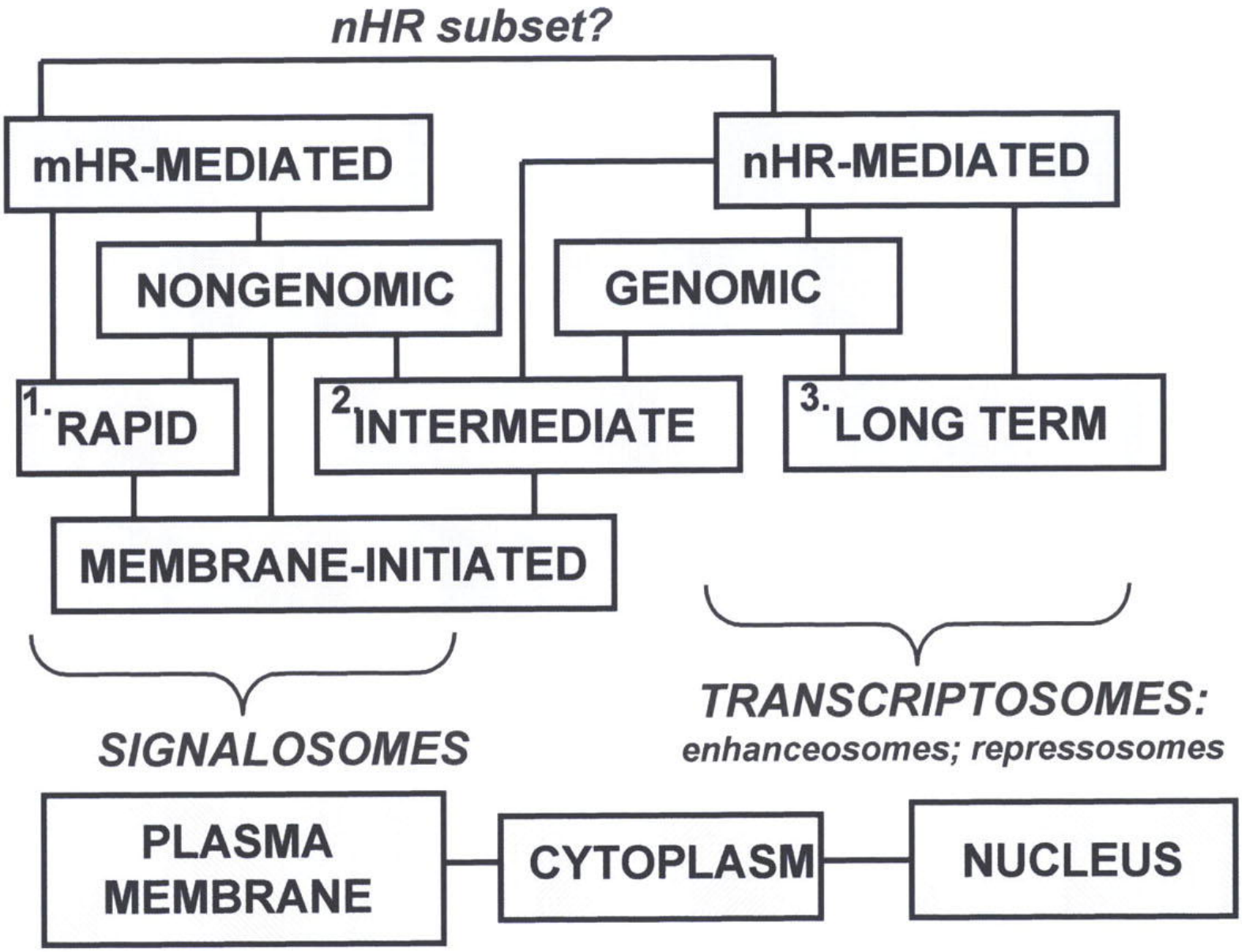
FIGURE LEGENDS

Figure 1: Schematic illustrating the relationship between rapid, intermediate, and long-term actions of steroid hormones on target cells. The terms are described in the text. Solid lines emphasize the extensive cross-talk that occurs among various receptors and signal transducers (signalosomes) that ultimately lead to changes in transcription (transcriptosomes), either positive (enhanceosomes) or negative (repressosomes).


nHR subset?
mHR-MEDIATED
nHR-MEDIATED
NONGENOMIC
GENOMIC
1. RAPID
2 INTERMEDIATE
3. LONG TERM
MEMBRANE-INITIATED
SIGNALOSOMES
TRANSCRIPTOSOMES:
enhanceosomes; repressosomes
PLASMA MEMBRANE
CYTOPLASM
NUCLEUS