

Multiple Actions of Steroid Hormones—A Focus on Rapid, Nongenomic Effects

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Abstract—According to the traditional model, steroid hormones bind to intracellular receptors and subsequently modulate transcription and protein synthesis, thus triggering genomic events finally responsible for delayed effects. Based upon similarities in molecular structure, specific receptors for steroids, vitamin D₃ derivatives, thyroid hormone, retinoids, and a variety of orphan receptors are considered to represent a superfamily of steroid receptors. In addition, very rapid effects of steroids mainly affecting intracellular signaling have been widely recognized that are clearly incompatible with the genomic model. These rapid, nongenomic steroid actions are likely to be transmitted via specific membrane receptors. Evidence for nongenomic steroid effects and distinct receptors involved is presented for all steroid groups including

related compounds like vitamin D₃ and thyroid hormones. The physiological and clinical relevance of these rapid effects is still largely unclear, but their existence in vivo has been clearly shown in various settings including human studies. Drugs that specifically affect nongenomic steroid action may find applications in various clinical areas such as cardiovascular and central nervous disorders, electrolyte homeostasis, and infertility. In addition to a short description of genomic steroid action, this review pays particular attention to the current knowledge and important results on the mechanisms of nongenomic steroid action. The modes of action are discussed in relation to their potential physiological or pathophysiological relevance and with regard to a cross-talk between genomic and nongenomic responses.

I. Introduction and Historical Development

For more than 30 years steroids have been known to be involved in various physiological responses with a primary focus on the genomic aspects of action. According to the classic genomic theory of action, steroid hormones bind to specific receptors, which are intracellular transcription factors, and exert positive or negative effects on the expression of target genes (Beato et al., 1996; Beato and Klug, 2000). These effects are characterized by a specific delay and a sensitivity toward inhibitors of transcription and translation, e.g., actinomycin D and cycloheximide. Intracellular steroid receptors have been thoroughly characterized and, finally, cloned; they are composed of a ligand-binding domain, a DNA-binding domain, and several transactivation functions distributed along the molecule (Evans, 1988; Beato, 1989; Fuller, 1991). In addition to the delayed genomic steroid actions, increasing evidence for rapid, nongenomic steroid effects has been demonstrated for virtually all groups of steroids, and transmission by so far hypothetical specific membrane receptors is very likely. Nongenomic effects on cellular function involve conventional second messenger cascades, including phospho-

lipase C (PLC²) (Civitelli et al., 1990), phosphoinositide turnover (Morley et al., 1992; Morelli et al., 1993), intracellular pH (Jenis et al., 1993; Wehling et al., 1996), free intracellular calcium ([Ca²⁺]_i) (de Boland and Nor-

² Abbreviations: PLC, phospholipase C; AR, intracellular androgen receptor; [Ca²⁺]_i, free intracellular calcium; CHO, Chinese hamster ovary; CREB, cAMP-responsive element-binding protein; CBP, CREB-binding protein; DAG, diacylglycerol; DHEA-S, dehydroepiandrosterone sulfate; DBD, DNA-binding domain; LBD, ligand-binding domain; AF, activation factor; NOS, nitric-oxide synthase; eNOS, endothelial NOS; ER, intracellular estrogen receptor; BSA, bovine serum albumin; cGMP, cyclic guanosine monophosphate; ERK, extracellular signal-regulated kinase; CNS, central nervous system; GABA, γ -aminobutyric acid; GR, intracellular glucocorticoid receptor; HML, human mononuclear leukocytes; HRE, hormone response element; 5-HT₃, 5-hydroxytryptamine type 3; MAP kinase, mitogen-activated protein kinase; mGR, membrane glucocorticoid receptor; MR, intracellular mineralocorticoid receptor; mPR, membrane progesterone-binding site; NF κ B, nuclear factor κ B; FITC, fluorescein isothiocyanate; NMDA, *N*-methyl-D-aspartate; OTR, oxytocin receptor; PC12 cells, pheochromocytoma cells; PKA, protein kinase A; PKC, protein kinase C; PPAR, peroxisome proliferator-activated receptor; PR, intracellular progesterone receptor; ROS, rat osteosarcoma cells; PAGE, polyacrylamide gel electrophoresis; SHBG, sex hormone-binding globulin; T₃, triiodothyronine; THDOC, tetrahydrodeoxycorticosterone; THP, tetrahydroprogesterone; VDR, intracellular vitamin D₃ receptor; 1 α ,25-(OH)₂D₃, 1 α ,25-(OH)₂-vitamin D₃; 1 β ,25-(OH)₂D₃, 1 β ,25-(OH)₂-vitamin D₃; VSMC, vascular smooth muscle cells.

man, 1990b; Wehling et al., 1990), and protein kinase C (PKC) (Sylvia et al., 1993). They are clearly incompatible with the involvement of genomic mechanisms. Interestingly, the history of research on rapid responses of steroid hormones actually predates the knowledge on the existence of nuclear receptors. In 1942, Hans Selye was the first to describe a rapid effect of progesterone, which following intraperitoneal application induces a prompt onset of anesthesia in rats (Selye, 1942). In 1963, acute cardiovascular effects of aldosterone in men were demonstrated (Klein and Henk, 1963). Peripheral vascular resistance and blood pressure increased within 5 min, while cardiac output significantly decreased, suggesting a nongenomic mechanism of action because of the short time frame. Almost simultaneously, Spach and Streeten (1964) reported *in vitro* effects of physiological concentrations of aldosterone on Na^+ exchange in dog erythrocytes. As a nucleus is absent, *in vitro* effects in these cells cannot be related to genomic mechanisms and, therefore, must be nongenomic in nature. Furthermore, rapid effects of glucocorticoids on isolated synaptosomes were demonstrated in the mid-1970s, being considered as cellular correlate for the long-known negative feedback mechanism between plasma cortisol and ACTH release occurring within a few minutes (Edwardson and Bennett, 1974). As another early example for a nongenomic steroid effect, Pietras and Szego (1975) showed rapid estrogen action on Ca^{2+} flux in endometrial cells in 1975. Despite these “old” observations, discussion about rapid, nongenomic mechanisms of steroid actions has emerged only recently (Gametchu, 1987; Wehling et al., 1987; Nemere and Norman, 1990).

In the following sections, important aspects of steroid action are summarized. Two sections on mechanisms of steroid actions in principle and the proteins that mediate them are followed by a condensed summary on nongenomic actions of each particular steroid group. Finally, clinical perspectives are discussed and an integrative model of steroid action is developed.

II. How Do Steroids Act?

A. Genomic Steroid Action

According to the common theory of steroid action, steroids modulate gene transcription by interaction with intracellular, nuclear receptors, which act as ligand-dependent transcription factors. Steroids regulate expression of various genes in a network-like manner and initiate complex events involved in nearly every aspect of vertebrate development and physiological responses (Evans, 1988; Beato et al., 1996; Beato and Klug, 2000).

The detailed characterization of steroid actions and mechanisms involved was the result of intensive long-term research on steroid hormones. In the beginning of the 20th century, abnormalities of embryonal development and various diseases had been associated with defects in steroid and thyroid hormone action (Guder-

natsch, 1912). The beginning of the modern era of steroid research is marked by a fundamental discovery by Clever and Karlson (1960), who investigated the puff reactions of chromosomes in larvae of insects. Injection of the steroid ecdysone induces changes in chromosomal structure within 2 h. These puff reactions disappear within 24 h, suggesting a link between steroid hormones and the activation of genes.

Subsequently, research has focused on the analysis of cellular and molecular mechanisms involved in related steroid actions on specific target tissues (for review see Beato, 1989; Fuller, 1991; Beato et al., 1996; Beato and Klug, 2000). Steroids bind to cognate, intracellular receptors representing a superfamily of steroid/thyroid/retinoid/orphan receptors. Interestingly, orphan receptors may have no ligands or as yet undiscovered ligands (Funder, 2000); they seem to open a particularly challenging field of future research.

These receptors act as transcription factors to regulate gene expression by recognizing palindromic hormone response elements (HRE) at the DNA after homo- or heterodimerization of the ligand-receptor complex. Subsequently, transcription is initiated in conjunction with the basal transcription complex, different coactivators, repressors, and transcription regulators (Beato and Klug, 2000). The ligand-dependent modulation of transcription by the ligand-receptor complex has been termed “genomic” and is sensitive to inhibitors of transcription and translation. The expression of steroid-induced genes is modulated at the protein level some hours after stimulation with the steroid, although immediate early genes are differentially expressed after aldosterone stimulation within 1 h (Verrey, 1998).

Unlike intracellular steroid receptors, membrane-bound receptors of other agonists (such as peptide agonists, catecholamines, or platelet-derived growth factor) affect cellular function by modulation of intracellular second messenger levels. In addition to these direct effects of second messengers, agonist-induced changes of intracellular messengers modulate steroid-induced transcription by an intracellular cross-talk. Thus, activation of cells by peptide agonists may modulate steroid-induced nuclear transcription by second messengers induced with an intrinsic ability to modulate nuclear transcription [e.g., cAMP (Nordeen et al., 1994)]. Furthermore, intracellular cross-talk may even occur in the absence of the steroid ligand. Epidermal growth factor activates the estrogen receptor (ER) α by signaling through the MAPK pathway, suggesting that MAPK directly phosphorylates the critical serine 118 of ER α (Bunone et al., 1996).

B. Nongenomic Steroid Action

In contrast to genomic steroid action, nongenomic steroid effects are principally characterized by their insensitivity to inhibitors of transcription and protein synthesis, and—representing the most obvious experimental

evidence—by their rapid onset of action (within seconds to minutes). These rapid effects are likely to be mediated through receptors with pharmacological properties distinct from those of the intracellular steroid receptors (see below). Discrepancies in pharmacological properties alone are not sufficient to support the hypothesis of separate receptor proteins for nongenomic action; however, this important issue is addressed in *Section III.B.*, and various evidence for the involvement of both classic and nonclassic receptor proteins in nongenomic signaling is given.

In the past two decades, a growing body of reports dealing with nongenomic steroid action has emerged, which reflects the increasing interest in this field. In these studies a variety of potential mechanisms thought to be involved in rapid steroid action has been described, suggesting that the mechanisms of rapid steroid signaling are not uniform. In this context, a classification of rapid steroid effects in distinct categories, relating to the mechanisms involved, has been proposed and discussed at the “First International Meeting on Rapid Responses to Steroid Hormones” in Mannheim, Germany, in 1998. This Mannheim classification scheme (Fig. 1) (Falkenstein et al., 2000) will help to adequately describe potential mechanisms involved in differential experimental settings and to facilitate the understanding of nongenomic steroid action. The scheme is divided into two major groups termed A (direct steroid action) and B (indirect steroid action), which are subsequently split into a nonspecific (I) and a specific (II) category. The latter is further divided into group a (classic steroid receptor involved) and b (nonclassic steroid receptor involved). In *Section III.*, examples for the categories AI, AIIa, AIIb, and BIIb are given. For categories BI and BIIa, no examples are known to date.

Each of the steroid and thyroid hormones displays its own facets of signaling and modulation of cellular functions. Specific nongenomic responses seem to depend on the type of steroid, cells, tissues, or species used. Nevertheless, signaling cascades share large homologies

with $[Ca^{2+}]_i$, PKC, PLC, cAMP, pH, MAP kinase, and other traditional second messengers playing major parts of variable, but similar, scores.

III. Steroid Receptors Mediating Genomic and Nongenomic Steroid Action

A. Receptors Responsible for Genomic Steroid Action

The concept that steroids are involved in the regulation of cell function was originally triggered by the above-mentioned observation that the steroid hormone ecdysone induces puffs in giant chromosomes of insects (Clever and Karlson, 1960). All steroid hormones, which are mainly formed in the gonads and adrenals of mammals, regulate a variety of functions in target cells equipped with the cognate steroid hormone receptors. Although steroid hormones and retinoic acid, vitamin D₃, and thyroid hormones are neither structurally nor biosynthetically related, receptors for steroids, retinoic acid, vitamin D₃, and thyroid hormones have been characterized as nuclear receptors or a superfamily of steroid and thyroid hormone receptors due to their close structural homologies (Evans, 1988; Beato and Klug, 2000).

1. *Structural Features of Steroid Hormone Receptors.* The human glucocorticoid receptor (GR), which has been cloned and expressed as one of the first steroid hormone receptors in the early 1980s, exists as a 777-amino acid, ligand-binding GR α displaying close homologies to the viral oncogen *erbA* and 742-amino acid β -isoform, which differs in the last 115 amino acids and does not bind glucocorticoids (Hollenberg et al., 1985). The binding characteristics of the GR are consistent with pharmacological properties of glucocorticoid-induced effects shown previously by *in vivo* and *in vitro* studies describing a high-affinity binding for the synthetic glucocorticoid dexamethasone and low-affinity binding for mineralocorticoids (Lee et al., 1988; Gottschall et al., 1991; Lemberger et al., 1994). Subsequently, the mineralocorticoid receptor (MR) (Arriza et al., 1987) and receptors for estradiol (ER) (Greene et al., 1986; Krust et al., 1986),

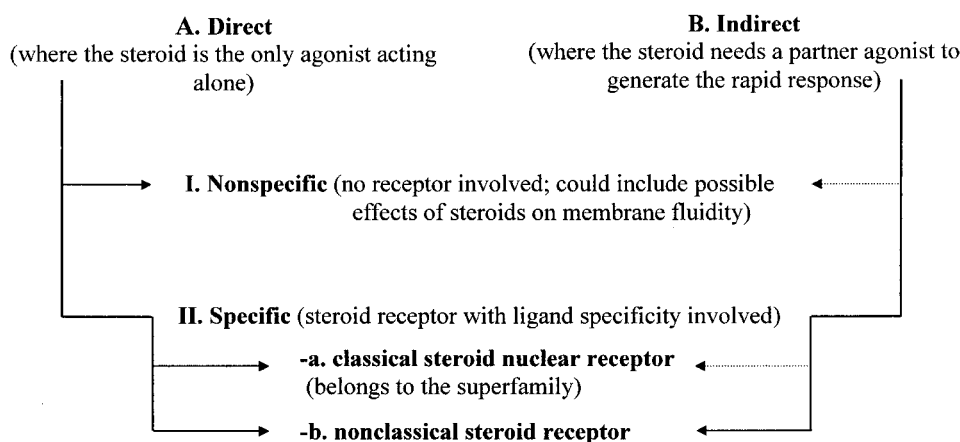


FIG. 1. Mannheim classification of nongenomic steroid actions. Solid arrows indicate examples for categories with examples given in the text. Dotted arrows indicate a hypothetical category with no example yet known. Reproduced with permission from Falkenstein et al. (2000).

progesterone (PR) (Loosfelt et al., 1986; Misrahi et al., 1987), androgens (AR) (Chang et al., 1988; Lubahn et al., 1988), vitamin D₃ (VDR) (McDonnell et al., 1987), retinoic acid (Petkovich et al., 1987), and thyroid hormone (Weinberger et al., 1986; Giguere et al., 1988) have been cloned, sequenced, and functionally expressed. The β -isoform of the GR has been regarded to be a cloning artifact for a long time; however, variants of steroid receptors and receptor isoforms generated by differential promoter usage have been described for nearly all steroid receptors (Kastner et al., 1990; Kuiper et al., 1996; Zennaro et al., 1997). However, the distinctive role of each of those variants is currently not known in detail.

Primary amino acid sequences of these receptors have been aligned on the basis of regions of maximum amino acid similarity. The nuclear receptors are structurally organized in different domains: a variable N-terminal region, a central, highly conserved, cysteine-rich DNA-binding domain (DBD), and a C-terminal ligand-binding domain (LBD). Furthermore, hormone-dependent transcriptional activation domains have been identified, which are embedded within the LBD and the N-terminal domain (Beato, 1989; Beato and Klug, 2000) (Fig. 2). More than 60 different gene products have been described that appear to belong to the nuclear receptor family on the basis of sequence identity, among them are receptors for the known hormones and, additionally, many structurally related gene products with unknown or no respective ligand ("orphan receptors"; Mangelsdorf and Evans, 1995).

The field of "reverse endocrinology" evolved. Historically, a new hormone has been discovered and characterized, and the partner receptor has been looked for,

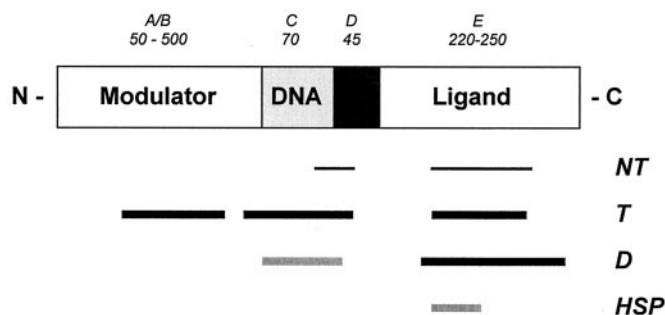


FIG. 2. General structure and functional organization of steroid and thyroid hormone receptors. Steroid receptors are structurally organized in different domains, which have been confirmed by results of cDNA cloning experiments: 1) a variable N-terminal region, which may have modulatory effects on transactivation (regions A/B, with a variable length of 50–500 amino acids). Most of the receptor antibodies described are directed against this part of the protein. 2) a well conserved cysteine-rich central domain, which exhibits cysteine residues compatible with the formation of zinc fingers (region C and D, with a length of ~70 and 45 amino acids). This part of the protein may be responsible for the DNA binding activity of the receptors (Evans, 1988). 3) a C-terminal domain, which is obviously responsible for hormone binding and nuclear translocation (region E, with a length of 220–250 amino acids). The functional assignment of amino acid sequences derived from cDNA cloning experiments is indicated below. NT, nuclear transcription; T, transactivation; D, dimerization; HSP, 90-kDa heat-shock protein binding. Modified according to Beato (1989).

whereas here the sequence information of an assumed receptor may be obtained, and the ligands binding to these orphan receptors are unknown and remain to be identified. Subsequently, a vitamin A metabolite 9-cis retinoic acid has been characterized as a high-affinity ligand for the three retinoic X receptor subtypes (Heyman et al., 1992). Furthermore, peroxisome proliferator-activated receptors (PPARs) have been detected as members of orphan nuclear receptors. Their name reflects the fact that PPARs are activated by chemicals that increase the number and size of peroxisomes in rodents (Issemann and Green, 1990). PPARs are regarded to be key regulators of glucose and lipid homeostasis (Lemberger et al., 1996; Kliewer et al., 1999). There is now compelling evidence that several of the orphans are a new generation of steroid receptors presumably revealing a broader biological role for steroid hormones than previously appreciated.

2. Genomic Steroid Hormone Action. It is assumed that the lipophilic steroid hormones enter the respective target cells by simple diffusion, although the matter of active transmembrane transport is still under debate but unsettled (Allera and Wildt, 1992). Steroid hormone receptors are associated with a complex of chaperone proteins in the unliganded state (Pratt and Toft, 1997; Defranco, 2000). Upon binding of steroids to the cognate steroid hormone receptor in the cytosol, the heat-shock protein Hsp 90 and the immunophilin Hsp 56, which maintain the receptors in an inactive form with high affinity for the steroid hormones, dissociate from the receptors (Pratt and Toft, 1997). This transformation of the steroid hormone receptor is associated with an increased affinity of the receptor to DNA and a decrease in complex size. The chaperones are probably necessary to keep the steroid hormone receptors functional (Godowski and Picard, 1989).

The cavity of the steroid hormone receptors, which is able to bind the steroid ligand, is created by the LBD and is covered by helix 12 of the LBD after hormone binding. Due to the relocation of helix 12 after hormone binding, coactivators are able to bind to respective parts of the LBDs (Fig. 2) as shown for ER α (Brzozowski et al., 1997). Although the selective ER modulator raloxifene binds to the ligand binding structure of ER α , the conformational change of the receptor leads to an orientation of helix 12, which is unable to bind respective coactivators (Brzozowski et al., 1997). The knowledge of the atomic structure of nuclear receptors before and after hormone binding will allow researchers to specifically design compounds that exhibit specific and efficient features to differentially modulate nuclear receptor function.

GRs and MRs translocate into the cell nucleus after hormone binding, probably due to the release of nuclear localization signals, whereas the majority of nuclear receptors, such as ER, AR, and PR, are located in the nucleus at equilibrium. Constitutively expressed nu-

clear localization signals of those receptors at the DBD are thought to be required for nuclear pore recognition, whereas secondary nuclear localization signals at the LBD domains are ligand-dependent (Guiochon-Mantel et al., 1991).

3. *Steroid Hormone-Responsive Elements.* After translocation into the nucleus, the ligand-receptor complex binds to palindromic DNA sequences. The receptors for glucocorticoids, mineralocorticoids, androgens, and progestins bind to the same HRE, which have been originally described as glucocorticoid response elements. HRE are hexanucleotide halves arranged as inverted repeats and separated by three nonconserved base pairs [AGAACAnnnTGTTCT (Beato, 1989)]. The sixth base pair of each half-palindrome is not well conserved, and its identity is not essential for specific binding (Scheidt et al., 1983). Initially it was believed that all members of the nuclear receptor family bind as homo- or heterodimers to the palindromic HREs in the promoter region of target genes, while each part of the HRE is recognized by one receptor monomer. However, the identification and characterization of the orphan estrogen-related receptors $ERR\alpha$ and $ERR\beta$ revealed that those receptors bind the DNA as monomers and homodimers (Johnston et al., 1997; Vanacker et al., 1999). Furthermore, the predominant form of $GR\alpha$ is monomeric in solution, whereas dimers of $GR\alpha$ are formed after binding of the ligand-receptor complex to an HRE. That the strength of a weak dimerization region within the DBD and the LBD is modified by cooperativity observed during DNA binding is obviously responsible for this dimerization (Eriksson et al., 1995).

Interaction of the steroid-receptor complex with the HRE is coordinated by the existence of two steroid hormone receptor-specific zinc fingers, a structure reminiscent but clearly different to the zinc finger motifs observed in the transcription factor IIIA of *Xenopus laevis* (Beato, 1989). The steroid hormone receptor-specific zinc finger is formed by four cysteines in the cysteine-rich region of the DBD and few amino acids at the adhering region (Fig. 2). While the proximal zinc finger (P-box) is responsible for specific interaction with the HRE, the distal box forms a weak dimerization area at the DNA-binding domain.

However, before interaction of the nuclear receptors with cognate HRE, the ligand-receptor complex must have the possibility to interact with the DNA, which is compacted into the chromatin. Genetic analysis has revealed that chromatin structure is essentially involved in gene regulation (Beato and Eisfeld, 1997). The DNA is packaged into nucleosomes, which consist of different histones around which the DNA is wrapped (van Holde et al., 1992). The question is how nuclear transcription factors gain access to the target sequences in the chromatin. Steroid hormone receptors can bind to regularly organized chromatin, probably due to the rotational orientation of HRE in nucleosomes, which has been ob-

served in vitro and in vivo (Pina et al., 1990). Human homologs of the yeast switching/sucrose nonfermenting complex, the so-called brahma, mediate the disruption of the nucleosome in an energy-dependent manner. The transactivation activity of the GR is weak in cells lacking brahma, whereas coexpression of the yeast switching/sucrose nonfermenting analog brahma in those cells significantly enhances the transactivation capacity of the GR (Muchardt and Yaniv, 1993, 1999). An interaction of the PR and the nucleosome-remodeling factor is involved in transactivation (Di Croce et al., 1999). Interestingly, some of the coactivators of steroid hormone receptors (see below) display histone-acetyltransferase activity. Acetylation of histones by those coactivators may largely reduce the affinity of histones to DNA, thus relieving the access of steroid hormone receptors to the HRE on the DNA (Beato and Klug, 2000). As other nuclear factors, such as the nuclear factor 1, cannot directly bind to their cognate targets in the DNA packed in nucleosomes, the DNA nucleotide sequence and its enzyme-dependent chromatin packaging may control the access of different transcription factors to their targets on the DNA (Beato and Eisfeld, 1997).

4. *Steroid-Induced Initiation of Transcription.* After binding of the respective DNA-recognition sites as homo- or heterodimers to the components of the basal transcriptional machinery and sequence specific coactivators, transcription starts or is down-regulated. Regulation of transcription not only depends on interaction with the consensus nucleotide sequence of the HRE, but also on interaction with the specific assembly of transcription factors and polymerases. Being sensitive to inhibitors of transcription and translation, related long-lasting physiological responses are classified as genomic actions of steroids. Among the earliest genomic steroid effects known is the increased rate of mouse mammary tumor virus long terminal repeat transcription in Ltk-aprt cells first seen within 7.5 min after glucocorticoid application (Groner et al., 1983). For mineralocorticoids, this effect starts within 30 min and peaks after 3 h in a feline renal cell line (Cato and Weinmann, 1988).

For initiation of transcription, the ligand-steroid receptor complex must interact with the transcription machinery of the nucleus. This interaction may be achieved by direct contact of the nuclear receptors with transcription factors, or indirectly by coactivators, mediators, and other factors facilitating transcription (Beato et al., 1996). Among the best characterized examples of coactivators for steroid-induced transcription are the steroid receptor coactivator 1 (Onate et al., 1995) and CREB-binding protein (CBP; Kamei et al., 1996). SRC-1 interacts with and enhances the human PR transcriptional activity without altering the basal activity of the promoter. Furthermore, the coexpression of SRC-1 reversed the ability of the ER to squelch activation by hPR (Onate et al., 1995), whereas ectopic expression of CBP or the related coactivator, p300, enhanced ER transcriptional

activity by up to 10-fold in a receptor- and DNA-dependent manner (Smith et al., 1996). The binding motif for these coactivators to the ligand-steroid receptor complex is released after the relocation of helix 12 of the LBD. However, the binding motif for coactivators is not revealed after binding of receptor antagonists to the steroid hormone receptors (Shiau et al., 1998). Interestingly, SRC-1 and CBP exhibit intrinsic histone-acetyltransferase activity, which may enable the transcription machinery to easily interact with the DNA of target genes.

Of major interest in terms of the reported cross-talk between ER and epidermal growth factor-dependent signaling is the observation that the interaction of the RNA helicase p68 with the N-terminal domain of ER α is potentiated after phosphorylation of the domain by MAP kinase, enhancing the transactivation activity of activation factor 1 (AF-1). However, it did not potentiate AF-1 or AF-2 of ER β , AR, retinoic acid receptor, or MR (Endoh et al., 1999).

In conclusion, the area of coactivators identified has extensively expanded during the recent years. However, most of those recently described coactivator complexes share a common and stable core, whereas small differences of their actions may provide the necessary specificity of coactivator function (Beato and Klug, 2000).

5. Alternative, Including Nontranscriptional Actions of Ligand-Steroid Hormone Receptor Complexes. In addition to the regulation of gene expression at the transcriptional levels, gene expression may be modulated by the interaction of nuclear receptors with sequence-specific transcription factors. For example, glucocorticoids affect the activity of NF κ B, an important modulator of cytokine-induced inflammation in at least two ways. Glucocorticoids genomically increase the expression levels of the inhibitor I κ B, which traps NF κ B in the cytoplasm (Auphan et al., 1995; Scheinman et al., 1995). In addition, GR interacts with p65, a transcriptionally active subunit of NF κ B, by protein-protein interaction (Ray and Prefontaine, 1994). Thus, glucocorticoids elicit distinct effects in different target tissues by direct actions at the transcriptional level and effects mediated by direct protein-protein interactions, which should be termed nontranscriptional activities of classic steroid receptors.

It was reported that progestins stimulate the c-Src kinase and the mitogen-activated protein kinase signal-transduction pathways via an interaction of ER with c-Src kinase (Migliaccio et al., 1998). Furthermore, direct interactions of steroids with nuclear DNA have been demonstrated (Hendry et al., 1977; Uberoi et al., 1985; Hendry, 1988); however, the physiological relevance of these observations remains yet to be determined. Thus, protein-protein and protein-DNA interactions independent of genomic actions of GR may additionally explain some of the specific steroid-induced effects.

B. Receptors Responsible for Nongenomic Steroid Action

1. Classic Intracellular Receptors (Classification AIIa). In various studies it has been demonstrated that classic steroid hormone receptors may be involved not only in genomic steroid action, but also in rapid nongenomic steroid effects. Rapid signaling is inhibited by classic antagonists of these receptors, as demonstrated for the AR, the ER, and the GR. As an example for category AIIa (Fig. 1), data supporting an involvement of ER in nongenomic estrogen effects are given below.

Immunohistochemical studies in GH3/B6 rat pituitary tumor cells using antibodies raised against epitopes of ER demonstrated positive staining at the plasma membrane (Pappas et al., 1995). These cells display nongenomic estrogen action as they rapidly release prolactin when treated with nanomolar concentrations of 17 β -estradiol. In particular, antibodies directed against a peptide representing the hinge region of ER, as well as other ER specific antibodies, each recognizing a unique epitope on ER, immunohistochemically label membrane proteins of immuno-selected GH3/B6 cells (Pappas et al., 1995). These data point to the existence of a membrane form of ER structurally similar to the classic intracellular ER, which exists in at least two subtypes, ER α and ER β . So far, abundant data could be obtained for ER α , whereas data on ER β are still sparse.

Evidence for a membrane localization of ER α could also be found recently in cultured hippocampal neurons (Clarke et al., 2000). Using isolated fetal rat hippocampal neurons, several antibodies directed against ER α showed positive membrane staining in nonpermeabilized neurons. In permeabilized hippocampal neurons, the staining for ER α could be found in the perinuclear area, but abundant labeling for ER α was detected throughout the cell, including the neurites. In the presence of 10 μ M antisense oligonucleotide directed against the translation start site of ER α , the immunoreactivity of ER α was reduced throughout the neurons, providing further evidence that the immunostaining was specific for ER α . Moreover, conventional and confocal microscopy showed that the antigen was localized predominantly in the extranuclear compartment, and detection of ER α in neurites suggests that the receptor is at least close to the plasma membrane (Clarke et al., 2000).

In early passage ovine fetal pulmonary artery endothelial cells, 17 β -estradiol stimulates the nitric-oxide synthase (NOS) activity within 5 min—an effect that could be completely blocked by the ER antagonists tamoxifen and ICI-182,780 (Lantin-Hermoso et al., 1997), but not by actinomycin D (Chen et al., 1999). The acute estradiol stimulation of NOS could be further increased by overexpression of ER α (Shaul et al., 1997). In addition, the acute response of endothelial NOS (eNOS) to 17 β -estradiol can be reconstituted in COS-7 cells trans-

fects with wild-type ER α and eNOS, but not by transfection with eNOS alone. Furthermore, inhibitors of Ca²⁺ influx, tyrosine kinases, or MAP kinases prevent the activation of eNOS by 17 β -estradiol, and 17 β -estradiol leads to a rapid ER α -dependent activation of MAP kinase (Shaul, 1999). This finding was confirmed recently by Russell et al. (2000), who reported that also in human umbilical vein endothelial cells, 17 β -estradiol activates the MAP kinase as well as cGMP synthesis and NO release, and that these effects could be triggered with membrane-impermeant forms of 17 β -estradiol. Again, these effects could be blocked by the ER antagonist ICI-182,780. Goetz et al. (1999) demonstrated that estrogen at concentrations as low as 1 pM induced a rapid translocation of NOS from the plasma membrane to perinuclear sites by a Ca²⁺-dependent, receptor-mediated mechanism. cGMP release could be rapidly increased by 17 β -estradiol, and this effect could be blocked by the ER antagonist ICI-164,384. In human umbilical vein endothelial cells, similar results have been obtained by using FITC-labeled 17 β -estradiol coupled to BSA as well as an antibody against ER. About 6 to 7% of cells contained the classic ER located at the surface of the cells (Caulin-Glaser et al., 1997). Latter authors demonstrated a rapid, estrogen-induced increase in the release of cGMP in these cells. Again, this increase could be blocked by the ER antagonist ICI-182,780 (Caulin-Glaser et al., 1997). In isolated hippocampal CA1 neurons, 17 β -estradiol can amplify kainate-induced currents, a protein kinase A (PKA)-dependent effect which cannot be blocked by ICI-182,780 (Gu et al., 1999).

Using peroxidase-conjugated estradiol, results of ligand blot analysis point to a specific estradiol-binding protein in human sperm. The same protein band could be detected by an antibody directed against the steroid binding domain of the classic ER (α H222). Functional analysis showed a rapid and sustained increase of [Ca²⁺]_i. These effects on [Ca²⁺]_i could also be obtained by use of the BSA-17 β -estradiol conjugate, which is incapable of penetrating the plasma membrane (Luconi et al., 1999).

A recent study has shown that in bovine aortic endothelial cells, as well, acute exposure of 17 β -estradiol (5 nM) increased NO production through ER α localized in specific plasma membrane domain caveolae. The 17 β -estradiol-stimulated NO production reached its maximum at 5 min before falling to near basal levels over the next 30 min. The rapid onset, the attenuation of the 17 β -estradiol response, and the observation that the effect was not accompanied by an increase of eNOS protein expression suggest that these effects were caused by a nongenomic action of 17 β -estradiol and do not require genomic eNOS up-regulation. The short duration of the NO increase suggests that 17 β -estradiol leads to an acute activation of eNOS followed by an inactivation afterwards. The mechanism that mediates this short response is still unclear. This effect could be

blunted by various agents that decrease [Ca²⁺]_i. The site of action is probably at the plasma membrane since BSA-conjugated 17 β -estradiol also increased the NO concentration. Furthermore, the pure ER α antagonist ICI-182,780 completely blocked estrogen-stimulated NO release (Kim et al., 1999). The suggestion that a version of the classic nuclear receptor ER α also exists in the plasma membrane is supported by findings that small numbers of both ER α and ER β were expressed in the plasma membrane of Chinese hamster ovary (CHO) cells transfected with both of the receptors (Razandi et al., 1999).

A membrane-associated 17 β -estradiol-binding protein could be characterized recently in rabbit uterus. Specific and saturable 17 β -estradiol-binding sites of high affinity were detected in uterine microsomes at higher concentrations than in cytosol. The stereoisomer 17 α -estradiol and the antiestrogen tamoxifen were less effective than 17 β -estradiol to compete with the radioactive ligand for binding to the membranes. Antibodies against the steroid binding domain were as effective as an inhibitor for cytosolic and membrane-specific radioligand binding. These findings are consistent with the existence of 17 β -estradiol membrane-binding proteins, which are structurally related to ER (Monje and Boland, 1999).

2. Nonclassic Steroid Receptors—No Coagonist Required (Classification AIIb). A wide array of nongenomic effects of steroids appear to be mediated through putative nonclassic membrane receptors with pharmacological properties that are clearly distinct from those of the classic intracellular steroid receptors. Although a divergent pharmacology does not prove the existence of distinct membrane receptors, it is one among other arguments to support this assumption. Other arguments include the existence of nongenomic steroid effects in cells or tissues devoid of the respective classic receptor [e.g., in cells from knockout animals as shown for MR and PR (see below)] and the insensitivity of rapid steroid effects to classic antagonists (e.g., spironolactone in the case of aldosterone). The ultimate proof would be the cloning and functional re-expression of an unrelated protein transmitting rapid steroid effects, which, however, has not been convincingly achieved for any steroid yet.

An example for this category, AIIb (Fig. 1), represents acute effects of 1 α ,25-(OH)₂D₃, which have been demonstrated in a variety of systems (Zanello and Norman, 1997a) (see also *Section IV.E.*). For example, subnanomolar amounts of 1 α ,25-(OH)₂D₃ have been found to rapidly (within 2 min) stimulate the intestinal Ca²⁺ transport in the perfused chick intestine (termed "transcaltachia") (Norman et al., 1993a). Moreover, 1 α ,25-(OH)₂D₃ (10⁻⁸ M) significantly increased MAP kinase phosphorylation, with the earliest response being detectable at 30 s (Song et al., 1998). None of these immediate effects requires gene transcription or protein synthesis (Farach-Carson and Ridall, 1998).

$1\alpha,25\text{-(OH)}_2\text{-vitamin D}_3$ [$1\alpha,25\text{-(OH)}_2\text{D}_3$] is a conformationally flexible molecule; therefore, a series of analogs locked in either the *cis* or the *trans* conformation have been used to assess the optimal shape for the nongenomic activity of the molecule. The *cis*-locked conformers activate the rapid, nongenomic pathways but bind poorly to the nuclear receptor and are only weak agonists for the genomic responses (Farach-Carson and Ridall, 1998; Song et al., 1998). In addition, a specific antagonist, $1\beta,25\text{-(OH)}_2\text{D}_3$, was found to be a potent inhibitor of transcalcemia but was unable to block the genomic effects of $1\alpha,25\text{-(OH)}_2\text{D}_3$ (Norman et al., 1993a).

These results suggest that the nuclear hormone D receptor is not involved in nongenomic $1\alpha,25\text{-(OH)}_2\text{D}_3$ -mediated effects and a distinct receptor may be responsible for its acute effects. In this context, a $1\alpha,25\text{-(OH)}_2\text{D}_3$ -binding site located in the basal-lateral membrane of vitamin D-replete chick intestinal epithelium has been described that was functionally correlated with transcalcemia. This protein exhibited saturable binding for [^3H] $1\alpha,25\text{-(OH)}_2\text{D}_3$ ($K_D = 0.72$ nM, $B_{\max} = 0.24$ pmol/mg protein) (Nemere et al., 1994). A functional correlation between the $1\alpha,25\text{-(OH)}_2\text{D}_3$ membrane-binding site and transcalcemia was observed in three experimental situations: 1) vitamin D deficiency, which suppresses transcalcemia, resulted in reduced specific binding of [^3H] $1\alpha,25\text{-(OH)}_2\text{D}_3$ to the basal-lateral membrane relative to corresponding fractions from vitamin D-replete chicks; 2) the $1\alpha,25\text{-(OH)}_2\text{D}_3$ membrane-binding site exhibited down-regulation of specific [^3H] $1\alpha,25\text{-(OH)}_2\text{D}_3$ binding following exposure to the nonradioactive ligand; and 3) the relative potencies of two “6-*s-cis*” analogs of $1\alpha,25\text{-(OH)}_2\text{D}_3$ [particularly $1\alpha,25\text{-(OH)}_2\text{-7-dehydrocholesterol}$ and $1\alpha,25\text{-(OH)}_2\text{-lumisterol}_3$] to bind to the $1\alpha,25\text{-(OH)}_2\text{D}_3$ membrane protein and their ability to initiate transcalcemia were congruent (Nemere, 1995). In a further set of experiments done with basal-lateral membranes of vitamin D-replete chick intestinal epithelium, a polyclonal antiserum (Ab99) directed against this membrane receptor was able to block the binding of the radioligand and to recognize a single protein band of 64.5 kDa in Western blot analyses (Nemere et al., 2000). A protein of similar size was also labeled by the affinity ligand [^{14}C] $1,25\text{-(OH)}_2\text{D}_3$ bromoacetate. The labeling was reduced in the presence of an excess of unlabeled secosteroid. The monoclonal antibody against the nuclear VDR (9A7) failed to detect an appropriate band in basal-lateral membrane fractions (Nemere et al., 2000). Similarly, an immunoreactive protein of 66 kDa was found in rat chondrocytes (Nemere et al., 1998). In the latter cells, Ab99 blocked the $1\alpha,25\text{-(OH)}_2\text{D}_3$ -dependent increase in PKC activity in chondrocytes, supporting the finding that the membrane receptor is involved in the initiation of $1\alpha,25\text{-(OH)}_2\text{D}_3$ -induced rapid nongenomic responses (Nemere and Farach-Carson, 1998).

The $1\alpha,25\text{-(OH)}_2\text{D}_3$ analog [^{14}C] $1\alpha,25\text{-(OH)}_2\text{D}_3$ bromoacetate was found to label a membrane protein in ROS24/1 cells that was identified to be annexin II (Baran et al., 2000). In addition, antibodies to annexin II blocked the vitamin D-induced increases in $[\text{Ca}^{2+}]_i$ and diminished the binding of $1\alpha,25\text{-(OH)}_2\text{D}_3$ to the protein in partially purified plasma membranes. However, these findings still await confirmation.

In summary, a substantial body of evidence now exists to indicate that at least some of the rapid $1\alpha,25\text{-(OH)}_2\text{D}_3$ -induced effects are transmitted by a membrane receptor distinct from the intracellular receptors belonging to the steroid and thyroid hormone superfamily. Further details of potential nonclassic steroid receptors will be discussed under the sections dedicated to particular steroid groups (see Section IV.).

3. Nonclassic Steroid Receptors—Coagonist-Mediated Steroid Action (Classification BIIb). Over the last decade, substantial experimental work has been carried out that investigated the metabolism and effects of various steroids in the brain and the central nervous system (CNS). Most effects are not mediated through nuclear steroid hormone receptors but through ion-gated neurotransmitter receptors. The potential of neuroactive steroids to modulate the γ -aminobutyric acid (GABA)_A receptor as allosteric coagonists or antagonists of GABA, or psychoactive drugs such as benzodiazepines and barbiturates, has attracted the most interest (category BIIb, Fig. 1). The mechanisms by which neuroactive steroids alter the excitability of GABAergic neurons depend on the specific structure of the GABA_A receptor with its subunits forming ligand-gated ion channels. Glycine-, nicotinic acetylcholine-, and 5-hydroxytryptamine type 3 (5-HT₃) receptors show remarkable homologies to the GABA receptors (Paul and Purdy, 1992; Lambert et al., 1995; Wetzel et al., 1998). GABA receptors are heterooligomeric proteins that contain a number of allosterically interacting binding sites for the neurotransmitter GABA, as well as for benzodiazepines and barbiturates. The first steroids shown to modulate the neuronal excitability by interaction with GABA_A receptors were $3\alpha,5\alpha$ tetrahydroprogesterone ($3\alpha,5\alpha\text{-THP}$) and $3\alpha,5\alpha$ tetrahydrodeoxycorticosterone ($3\alpha,5\alpha\text{-THDOC}$) (Majewska et al., 1986). These steroids are potent barbiturate-like ligands of the GABA receptor-chloride ion channel complex. At concentrations between 100 nM and 10 μM , both steroids inhibit binding of the convulsant *t*-butylbicyclo-phosphorothionate to the GABA receptor complex and, as coagonists at the GABA_A receptor, increase the binding of flunitrazepam. They also stimulate chloride uptake into isolated brain vesicles and potentiate the inhibitory actions of GABA in cultured rat hypothalamic neurons (Wetzel et al., 1999). In contrast to the pharmacological activity of benzodiazepines, which varies with the α -subunit composition and requires the presence of a γ -subunit, the effects of neuroactive steroids do not depend on such strictly defined

basic requirements for their structure-activity relationship (Puia et al., 1990). Studies investigating this relationship were able to delineate the presence of a 3α -OH group within the A-ring of neuroactive steroids as the crucial determinant for a positive allosteric interaction at the GABA_A receptor to enhance GABA or benzodiazepine action (Gee et al., 1988; Paul and Purdy, 1992). All 3β -hydroxysteroids that have been investigated so far seem to be inactive in increasing GABA_A receptor-mediated Cl⁻ conductance or flux (Purdy et al., 1990; Paul and Purdy, 1992), leading to the conclusion that 3α -hydroxysteroids have a distinct stereoselectivity at the GABA_A receptors. In contrast, the 3α -reduced pregnane steroids dehydroepiandrosterone sulfate (DHEA-S) and pregnenolone sulfate have been shown to exert GABA-antagonistic properties at the GABA_A receptor (Lambert et al., 1995; Rupprecht, 1997; Shen et al., 1999). This allosteric antagonism to GABA at the receptor, as well as the described coagonistic activity of other neuroactive steroids, confers a multitude of functional effects. These are briefly discussed below but have been extensively reviewed elsewhere (Paul and Purdy, 1992; Lambert et al., 1995). In addition to the 3α -OH group within the A-ring of neurosteroids, there may be other components to the structure activity relationship of neurosteroids at the GABA_A receptor. It has recently been shown that 6-oxa analogs of the neurosteroid 3α -hydroxy- 5α -pregnan-20-one, which do not possess the carbon atom 6 within the B-ring, have an approximately 100-fold reduced potency for modulating flunitrazepam binding to the GABA_A receptor compared to their natural carbon analogs (Nicoletti et al., 2000). Certainly, the field of structure-activity relationship is still wide open for neurosteroids, and the complex interaction of neurosteroids with the GABA_A receptor needs further in depth investigation.

Research has focused on establishing a specific steroid-binding site on the GABA_A receptor, and evidence has accumulated that steroid recognition sites reside on the GABA_A receptor and not in the bilayer surrounding it. In *Xenopus* oocytes that were nonresponsive to the modulatory actions of steroids, a temporary expression of GABA_A receptor subunits can cause steroid sensitivity (Shingai et al., 1991), even though desensitization did not show stringent stereoselectivity (Woodward et al., 1992). In addition, $3\alpha,5\alpha$ -dehydroprogesterone has been shown to modulate ligand binding to solubilized GABA_A receptors in a manner consistent with ligand binding of membrane-bound receptors (Giusti et al., 1993).

The immense possibilities for GABA_A receptor heterogeneity due to multiple isoforms of each subunit allow for a heterogeneous population of GABA_A receptors to be present in the CNS (Burt and Kamatchi, 1991; Sieghart, 1992; Olsen and Sapp, 1995). It may well be that different combinations of GABA_A receptor subunits compose GABA_A receptors with unequal sensitivities to the co-

agonistic potential of steroids. Although it has been proposed that GABA_A receptor function is modulated by steroids independently of the subunit formation of the receptor (Puia et al., 1990), other investigators were able to demonstrate that the efficacy of steroids to modulate GABA_A receptors is at least partly specific to the subunit composition of the receptor (Lan et al., 1991; Shingai et al., 1991; Zaman et al., 1992). This hypothesis is further supported by observations of possible regional differences in sensitivity to steroid actions within the CNS that may depend on varying subunit composition (Canonaco et al., 1993). The mechanisms by which these differences develop, however, have not yet been clearly defined. It is possible that GABA_A receptor subunit expression is regulated by the hormonal environment and that neuroactive steroids themselves are involved in this complex issue (Cooper et al., 1999). This concept is substantiated by the finding that the progesterone metabolites allopregnanolone and allotetrahydrodeoxycorticosterone modulate GABA_A receptor plasticity during pregnancy and after delivery in rats (Concas et al., 1999). For so far unknown reasons, the effects of neurosteroids may also depend on the particular CNS region, possibly due to the presence or absence of "cofactors" that researchers have just begun to identify. In the hippocampus, neurosteroid action has been proposed to depend on NO (Mehta and Ticku, 1999). When NO production was inhibited, the ability of pregnenolone to potentiate the muscimol binding in the rat hippocampus was markedly diminished.

However, one of the major drawbacks of research on neurosteroid effects on GABA_A receptor functionality is the lack of information from studies in humans. Still, on the basis of presently published results, an integral concept may be developed by which steroids alter brain function via the GABA_A receptor that can be extended to the physiological and pathophysiological context in humans.

4. No Receptor Involved—Direct Nongenomic Action (Classification AI). In addition to the above-mentioned specific receptor-mediated actions, direct steroid-membrane interactions occurring without receptor involvement have been described that alter physicochemical membrane properties such as the fluidity and the microenvironment of membrane receptors. This intercalation of steroids in phospholipid bilayers may occur at high, nonphysiological steroid concentrations. The corresponding effects are termed as nonspecific, nongenomic steroid actions (classification AI, Fig. 1).

Willmer (1961) proposed that steroids could be inserted into bilayers of cellular membranes, thereby affecting their fluidity. Accordingly, effects of micromolar concentrations of estradiol and progesterone on membrane fluidity have been shown in various tissues or cells, such as breast cancer (Clarke et al., 1990), vaginal epithelial cells (Reddy et al., 1989), and human spermatozoa (Shivaji and Jagannadham, 1992). In the latter,

interactions of progesterone, 17α -hydroxyprogesterone, testosterone, and estradiol with synthetic membrane vesicles and native spermatozoan membranes have been examined by light scattering and fluorescence spectroscopy. The results indicated that progesterone aggregates membrane vesicles, decreases the fluidity of membranes, induces fusion of membrane vesicles, and renders them permeable to hydrophilic molecules such as carboxyfluorescein. In this study optimal results were observed at a progesterone concentration of $\sim 30 \mu\text{M}$. In contrast, similar concentrations of testosterone and estradiol had very little effect on membrane fluidity, aggregation, fusion, and leakage. Thus, steroid specificity reflecting variable lipophilicity and polarity may be apparent even in the absence of receptor proteins. In general, nonspecific steroid actions can be expected at supramicromolar and, therefore, nonphysiological concentrations. Nevertheless, $1\alpha,25\text{-(OH)}_2\text{D}_3$ has been described to influence growth zone cell membrane fluidity in rat chondrocytes even at nanomolar concentrations (Swain et al., 1993).

However, in most instances, steroid concentrations required to elicit these effects are not achieved physiologically; thus the relevance of these effects is questionable. As a general rule, nonspecific steroid effects must be expected for all steroids at concentrations $\geq 10 \mu\text{M}$.

IV. Steroid Groups

A. Gonadal Steroids

1. Progesterone.

a. Rapid Effects of Progesterone. Since the pioneering work of Selye in 1942, which has led to the development of a variety of steroidal anesthetics, a growing number of reports dealing with rapid, nongenomic actions of progesterone has been published.

An extensive amount of work has been done with regard to the action of progesterone on amphibian oocyte maturation, demonstrating several intracellular signal transduction systems to be involved. Many of the progesterone-induced changes associated with meiosis also occur in enucleated oocytes, suggesting nongenomic effects of the steroid (Morrill and Kostellow, 1999). In *Rana pipiens* oocytes, progesterone triggers a transient release of Ca^{2+} from the oocyte surface within the first few seconds, followed by a decrease in intracellular cAMP (Kostellow et al., 1980) and a transient rise in cGMP (Kostellow and Morrill, 1980). Progesterone was also found to rapidly activate a series of reactions that generate DAG transients (Morrill and Kostellow, 1999).

In addition to oocytes, rapid progesterone effects on spermatozoa have been intensively studied not only in sperm from humans but also from other mammals, such as mice, (Herrero et al., 1997; Purohit et al., 1998), stallions (Cheng et al., 1998), hamsters (Llanos and Anabalon, 1996), and dogs (Sirivaidyapong et al., 1999). Because these effects occur within minutes after addi-

tion of the steroid, and because the intracellular PR could not be detected in human spermatozoa (Castilla et al., 1995; Luconi et al., 1998a), progesterone action in sperm is likely to be mediated by a pathway distinct from the genomic one (Baldi et al., 1998; Blackmore, 1998).

In many studies done with different experimental approaches (including flow cytometry, indirect immunofluorescence, and transmission electron microscopy), it has been shown that progesterone is one of the physiological stimuli of the sperm acrosome reaction (Osman et al., 1989; Meizel and Turner, 1991; Bronson et al., 1999). This effect of progesterone is due to its ability to induce a very rapid increase of intracellular $[\text{Ca}^{2+}]_i$ occurring within seconds after addition of the steroid (Baldi et al., 1991; Turner et al., 1994). Progesterone-induced $[\text{Ca}^{2+}]_i$ increase was found to be dose-dependent, with the smallest response at 1 nM and a maximum at concentrations of 1 to $10 \mu\text{M}$ (Blackmore et al., 1990), and is not blocked by RU-486, a potent antagonist of the intracellular PR (Baldi et al., 1991). These high concentrations of progesterone are present in the cumulus matrix surrounding the oocyte, which has to be passed by the spermatozoa to reach the zona pellucida (Baldi et al., 1999). The increase in $[\text{Ca}^{2+}]_i$ seems to involve influx of extracellular Ca^{2+} because it can be abolished by removal of the ion in the external medium with EDTA. The type(s) of Ca^{2+} channels mediating the increase in $[\text{Ca}^{2+}]_i$ are not known at present. However, Ca^{2+} influx was found to be partially inhibited by Ni^{2+} and La^{3+} , whereas verapamil and diltiazem were ineffective in blocking the effect, suggesting that L-type Ca^{2+} channels are unlikely to be involved (Blackmore et al., 1991). In addition to progesterone, also 17α -hydroxyprogesterone was described to generate a rapid Ca^{2+} response in sperm (Blackmore et al., 1990).

Moreover, progesterone has been shown to rapidly stimulate Cl^- (Turner and Meizel, 1995) and Na^+ fluxes (Foresta et al., 1993; Patrat et al., 2000) in spermatozoa. Progesterone has also been linked to other signal transduction mechanisms in spermatozoa. The steroid was found to rapidly stimulate phosphatidylinositol 4,5-bisphosphate hydrolysis, leading to formation of DAG and IP_3 , which is presumably due to an activation of Ca^{2+} -dependent PLC (Thomas and Meizel, 1989). Moreover, progesterone has been suggested to stimulate phospholipase A_2 activity in capacitated spermatozoa (Baldi et al., 1993; Roldan and Vazquez, 1996). Furthermore, a stimulation of tyrosine phosphorylation (Luconi et al., 1995; Martinez et al., 1999) and an involvement of p42 extracellular signal-regulated kinase (Luconi et al., 1998b) have been described in rapid progesterone signaling. Recently, evidence for participation of PKA in the progesterone-initiated acrosome reaction has been presented (Harrison et al., 2000).

In addition to producing rapid effects in reproductive tissues, progesterone has also been found to rapidly act

in several other tissues or cells. As for other steroids, rapid Ca^{2+} fluxes in response to progesterone were demonstrated in pig granulosa cells, which were unaffected by RU-486. Progesterone (0.1 pM–1 nM) triggers an immediate (within 5 s) and transient peak in $[\text{Ca}^{2+}]_i$, followed by a sustained plateau phase. This response involves both Ca^{2+} release from intracellular stores and Ca^{2+} influx, and it appears to be mediated by a pertussis toxin-insensitive G-protein (Machelon et al., 1996; Lieberherr et al., 1999).

An involvement of nongenomic progesterone signaling in maintaining pregnancy by depressing the uterotonic action of the peptide hormone oxytocin was suggested in a recent study by Grazzini et al. (1998). Progesterone, but not the progesterone metabolite 5β -pregnane-3,20-dione, inhibits oxytocin binding to rat uterine membranes containing the oxytocin receptor (OTR), a member of the G-protein-coupled receptor family. This effect was also found in CHO cells expressing recombinant rat OTR. The inhibition constants of both effects were 16 and 15 nM, respectively. Moreover, application of progesterone (10 nM–1 μM) to these cells caused an inhibition of the oxytocin-induced Ca^{2+} response. As circulating progesterone concentrations in the rat reached 500 nM during pregnancy, the effective progesterone concentrations were within physiological range (Grazzini et al., 1998). Interestingly, in CHO cells expressing recombinant human OTR, no inhibition of oxytocin binding by progesterone (up to 10 μM) was observed, but 5β -pregnane-3,20-dione inhibited oxytocin binding with an inhibition constant of 32 nM (Grazzini et al., 1998). In contrast to these findings, Burger et al. (1999) needed ~6000-fold higher concentrations of 5β -pregnane-3,20-dione to reduce oxytocin binding in CHO cells expressing recombinant human OTR. In these cells a maximal reduction in the oxytocin-induced Ca^{2+} signals was found only at nonphysiological progesterone concentrations (160 μM). Because the results of Grazzini et al. (1998) would have major impact in this regard, their confirmation is crucial but still not available.

A variety of other rapid progesterone effects have been demonstrated; however, they occur at nonphysiologically high steroid concentrations, rendering their relevance questionable. For example, progesterone at micromolar concentrations induces a dose-dependent relaxation of rat saphenous artery segments (precontracted with norepinephrine) and rat uteri (precontracted with KCl) within 10 min (Cabral et al., 1994; Gutierrez et al., 1994; Kakucs et al., 1998). In a similar manner, progesterone dose dependently decreases the contractile activity of murine jejunum (Oh et al., 1998). Intravenous progesterone (200 μg) significantly increased lordosis of ovariectomized, estradiol-primed mice within 10 min, an effect seen in both PR knockout and wild-type mice (Frye et al., 1992; DeBold and Frye, 1994; Frye and Vongher, 1999).

By the use of the whole cell patch-clamp technique, relatively high concentrations of progesterone (50 μM) have been described to dose dependently decrease Ca^{2+} currents in a human intestinal smooth muscle cell line (Bielefeldt et al., 1996). In rat hepatocytes, the addition of similar quantities of progesterone (1–100 μM) induces a rapid (within minutes) and completely reversible depolarization of the cell membrane, paralleled by a decrease of K^+ selectivity and an increase of cell membrane resistance (Waldegger et al., 1995). Furthermore, rapid electrophysiological effects of progesterone have been described in thymulin-secreting epithelial cells (Head et al., 1999), Leydig cells (Rossato et al., 1999), natural killer cells (Mandler et al., 1993), and CA1 hippocampal neurons (Joels and Karst, 1995).

b. Progesterone Receptors for Rapid Signaling. Membrane-binding sites for progesterone have been described and at least in part characterized in tissues or cells exhibiting nongenomic progesterone actions, thus pointing to a link between putative membrane receptors and rapid steroid effects. For *Xenopus laevis* oocytes, membrane-binding sites for R5020, modulation of cell signaling (cAMP, $[\text{Ca}^{2+}]_i$), and physiological effects (oocyte maturation) were described in the early 1980s (Wasserman et al., 1980; Blondeau and Baulieu, 1984). However, it should be mentioned that the synthetic steroid R5020 has both gestagen and glucocorticoid activities. In a different study, the binding of progesterone was shown in membranes from frog oocytes (Kostellow et al., 1980).

In addition to the rapid nongenomic actions of progesterone in the ovary, specific progesterone membrane-binding sites have been described in luteal membranes of several species. However, binding of [^3H]progesterone seems to occur only in the presence of digitonin (Menzies and Bramley, 1994; Rae et al., 1998; Menzies et al., 1999).

The existence of progesterone membrane-binding sites in several regions of the brain has been demonstrated by the use of iodinated progesterone-BSA exhibiting K_d values in the nanomolar range (Ke and Ramirez, 1990; Caldwell et al., 1995; Ramirez et al., 1996). Photoaffinity labeling experiments in mouse brain membranes with a progesterone analog detected four protein bands with apparent molecular masses ranging from 29 to 64 kDa (Bukusoglu and Krieger, 1994).

In rat hepatocytes, two progesterone membrane-binding sites have been described with K_d values of 9.5 and 50.7 nM, respectively (Trueba et al., 1990).

In search of specific steroid membrane-binding sites, our group was able to characterize two membrane progesterone-binding sites (mPR) from porcine liver microsomes with apparent K_d values of 11 and 286 nM, respectively. After purification, the maximum capacity of binding corresponds to the enrichment of polypeptides with relative molecular masses of 28 and 56 kDa, with

the 56-kDa protein possibly representing a dimer of the 28-kDa protein (Meyer et al., 1996). Under native conditions, only one particular membrane-binding protein complex of ~200 kDa was found, displaying progesterone binding activity. This finding has led to the hypothesis that the native mPR may be an oligomeric protein complex, composed at least in part of the 28- and 56-kDa proteins (Meyer et al., 1998a). Immunocytochemical localization studies and subcellular fractionation experiments provide evidence for the localization of the protein to endomembranes [endoplasmic reticulum, Golgi apparatus (Falkenstein et al., 1998)]. These data are still compatible with the assumption that the progesterone-binding protein may serve as an alternative nongenomic receptor for progesterone because the hydrophobicity of steroids allows for rapid entrance into cells through the plasma membrane, which in addition may be facilitated by hypothetical carrier proteins.

Recently, it has been found that microsomes from intact bovine lens epithelium bound progesterone with high affinity ($K_d \sim 75$ nM). Interestingly, Western blotting analyses of microsomes from bovine lens epithelial cells using a mPR-specific antibody indicated a microsomal 28-kDa protein that contained a N-terminal sequence that is very similar to mPR (Cenedella et al., 1999). Sequence analyses of cloned mPR exhibit no significant identity to the intracellular PR or any protein with known function so far (Falkenstein et al., 1996; Gerdes et al., 1998). Heterologous expression of the mPR-cDNA in CHO cells leads to an increase in microsomal progesterone binding capacity compared to microsomes from mock-transfected CHO cells. Recombinant mPR clearly displayed similar progesterone specificity compared to the native protein from porcine liver (Falkenstein et al., 1999).

Several studies have shown that nongenomic progesterone action in spermatozoa involves the binding of the steroid to specific receptors. Experiments done with membrane impermeable agents, BSA-conjugated progesterone, and progesterone-BSA-fluorescein isothiocyanate point to a direct interaction of progesterone with the sperm plasma membrane (Blackmore and Lattanzio, 1991; Meizel and Turner, 1991; Tesarik et al., 1992). Different types of membrane receptors are thought to be involved in rapid signaling (Revelli et al., 1998); by the use of progesterone-11 α -glucuronide-[¹²⁵I]iodotyramine as a tracer and ligand blot analysis of sperm total lysates, two progesterone-binding proteins with apparent molecular masses of 54 and 57 kDa were detected that are likely to be involved in the rapid progesterone effects in human sperm (Luconi et al., 1998a). In experiments using a monoclonal antibody raised against the steroid binding domain of the intracellular human PR, an inhibition of progesterone-induced Ca^{2+} influx and the acrosome reaction was shown. In addition, this antibody recognized a protein band with a molecular mass of 50 to 52 kDa and a minor band of 46 kDa (Sabeur et al., 1996).

In another study with the same antibody, proteins with molecular masses of 54 and 57 kDa were labeled (Luconi et al., 1998a). In contrast, an antibody directed against the DNA-binding domain of PR did not detect any specific band (Luconi et al., 1998a), confirming earlier experiments which demonstrated that PR is not present in human sperm (Castilla et al., 1995; Sabeur et al., 1996).

Similar to our findings in porcine liver microsomes (see above), proteins with molecular masses of 25 to 30 kDa and 50 to 60 kDa were detected in human sperm extracts by the use of antibodies against the rat homolog of mPR (25-Dx) (Selmin et al., 1996; Blackmore, 1999). Therefore, it is very likely that a progesterone membrane-binding protein with at least partial homology, if not identity, to mPR is present in human sperm.

To determine whether mPR not only binds progesterone but is also involved in nongenomic steroid action, rapid progesterone-induced Ca^{2+} fluxes in human sperm were analyzed in the presence or absence of a mPR-specific antiserum by our group (Falkenstein et al., 1999). Human spermatozoa incubated with the specific antibody exhibited a significantly reduced progesterone-induced Ca^{2+} increase compared to the corresponding preimmune serum (Fig. 3). These data are in line with recent findings indicating that the same mPR-specific antibody inhibits the progesterone-initiated acrosome reaction by 62.1% (Buddhikot et al., 1999). These inhibitory effects caused by the mPR-specific antibody imply that a progesterone membrane-binding protein with close similarity, if not identity, to mPR is involved in rapid progesterone action in human sperm.

Interestingly, the rat analog of mPR (25-Dx) (Selmin et al., 1996) was suggested to be behaviorally relevant. In a study done in the ventromedial hypothalamus, 25-Dx expression was repressed by progesterone after estradiol priming of ovariectomized rats. The expression of 25-Dx was found to be sexually dimorphic, and higher levels were seen in female PR knockout mice than in their wild-type littermates. These findings suggest a mechanism in which the activation of the intracellular PR represses the expression of 25-Dx (Krebs et al., 2000).

In summary, it is important to note that from the current knowledge nongenomic progesterone action does not exhibit an array of effects whose width is comparable to that of other steroids. In addition to its actions in the brain (see *Section IV.D.*), rapid effects of the steroid are mainly found in oocytes or sperm. In the latter, nongenomic progesterone actions seem to have a great impact on priming of spermatozoa for the fertilization process, and a relative abundance of reports dealing with progesterone membrane receptors in sperm exists.

2. Estrogens.

a. Rapid Effects of Estrogens. During the past years, a considerable number of studies describing effects of estrogens in various tissues that can be attributed to nongenomic estrogen signaling have been accumulated.

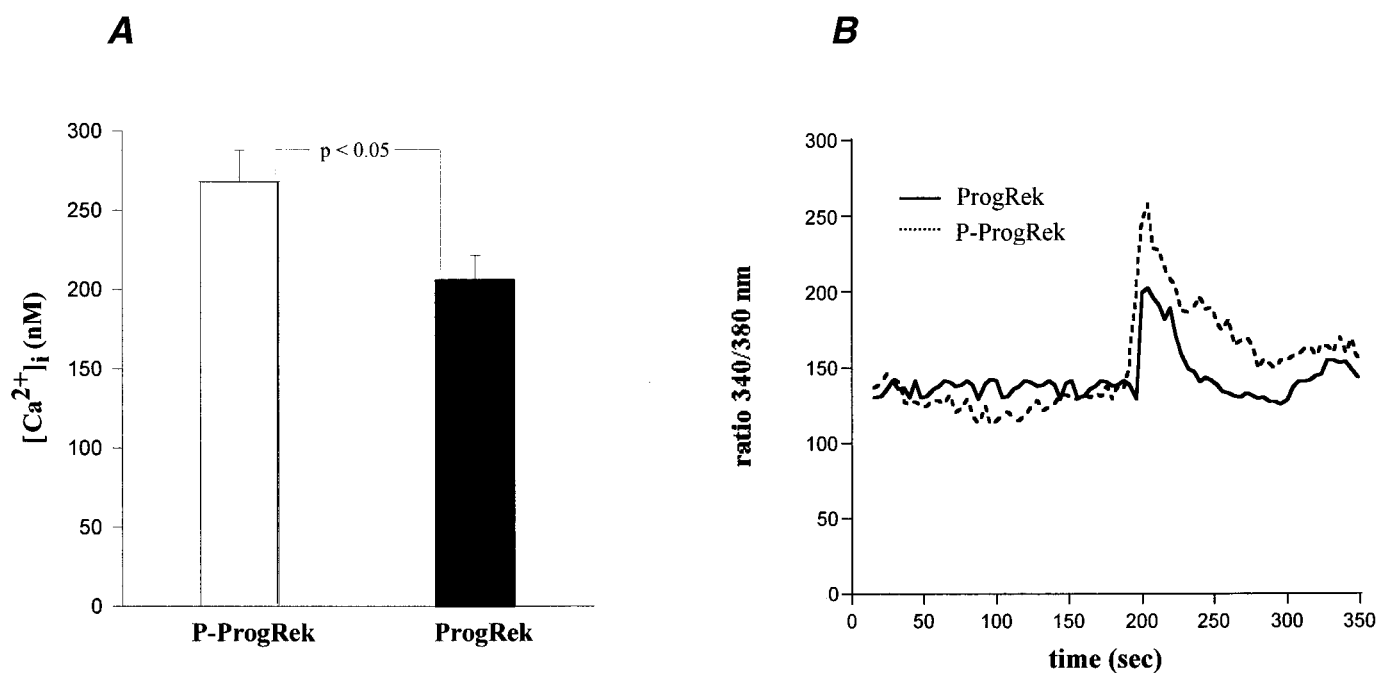


Fig. 3. Progesterone-induced Ca^{2+} increase in human sperm in the presence of a 1:10 dilution of a mPR-specific antiserum (ProgRek) or preimmune serum (P-ProgRek). $P < .05$ as indicated. A, Ca^{2+} response, mean of $n = 116$ cells (8 experiments). B, Ca^{2+} lanes in a typical experiment. Reproduced with permission from Falkenstein et al. (1999).

One focus is on the investigations of the impact of estrogens on the CNS. Estrogens are capable of modulating the physiology of nerve cells within seconds after application (Nabekura et al., 1986; Zakon, 1998). The rapid time course and the pharmacological profile of these effects with regard to the insensitivity to transcriptional and translational inhibitors lead to the assumption that genomic effects are not involved. It is now well established that estrogens act via interaction with specific receptors at the plasma membranes of neurons (Towle and Sze, 1983; Pappas et al., 1995; Ramirez et al., 1996), followed by the activation of intracellular signal transduction pathways. Recent findings have shown that estrogens stimulate the formation of cAMP (Gu and Moss, 1996), the phosphorylation of CREB (Zhou et al., 1996), and the formation of IP₃ (Favit et al., 1991) and activate the MAP kinase signaling pathway in a neuroblastoma cell line (Watters et al., 1997).

Previous results indicate that estrogens influence the differentiation of mouse midbrain dopaminergic neurons via nongenomic mechanisms, which include the interaction with a membrane receptor coupled to intracellular signaling pathways. Estrogens enhance the dopamine synthesis (Pasqualini et al., 1995) and release (Becker, 1990), modify basal firing rates (Chiodo and Caggiula, 1980), and lead to rotational and stereotypic behavior in adult rodents (Becker, 1990) within seconds after application. A recent study showed that treatment with 17 β -estradiol or BSA-estrogen conjugate increased neurite growth and arborization of dopaminergic neurons that could be inhibited by antagonists of cAMP/PKA and Ca^{2+} signaling pathways but not by the ER antagonist

ICI-182,780 (Beyer and Karolczak, 2000). Estrogens provoke a rapid Ca^{2+} release from intracellular stores in cultured embryonic dopaminergic neurons via the IP₃ signaling pathway; these effects are steroid-specific and localized to the membrane receptor (Beyer and Raab, 1998). Moreover, 17 β -estradiol stimulates the phosphorylation of CREB in midbrain dopaminergic cells as measured by quantitative double-labeling immunocytochemistry and gel shift assay. Simultaneous treatment with inhibitors of the cAMP/PKA or Ca^{2+} pathways antagonizes this effect, but treatment with the ER antagonist ICI-182,780 does not. These data demonstrate that nongenomic estrogen actions are involved in the neuronal differentiation of the mammalian brain (Beyer and Karolczak, 2000).

In addition to the effects of estrogens on brain differentiation, estrogens seem to have an important impact on the endocrinological function of the brain. Treatment of rat pituitary GH3/B6/F10 cells with 17 β -estradiol (1 nM) increases prolactin release after 3 to 6 min. Different types of antibodies directed against ER α that are too large to diffuse into cells were tested for their impact on ER α -mediated prolactin release. An antibody (Ab R4) against a hinge region of ER α increases prolactin release in a time- and concentration-dependent manner. Ab H151 targeted against a different hinge region epitope decreases prolactin release and blocked the effects of 17 β -estradiol. The results of this study emphasize the modulatory effects of 17 β -estradiol in prolactin release (Norfleet et al., 2000).

One of the first investigations about nongenomic actions of estrogens has been carried out in the male and

female reproductive system. In cultured endometrial cells, 17β -estradiol rapidly (<10 min) stimulates Ca^{2+} influx (Pietras and Szego, 1975). In ovariectomized rats, the intravenous injection of 17β -estradiol modifies cell morphology within 1 min, increasing the number and clustering of microvilli in the endometrial cells and the luminal surface ciliation (Rambo and Szego, 1983). These results could be confirmed by ultrastructural analysis and supplemented by findings that 17β -estradiol-induced structural changes are biphasic: they appear to be secondarily enhanced after 7 min (Rambo and Szego, 1983). Szego (1975) demonstrated that in ovariectomized rats 17β -estradiol elicits micropinocytotic activity in endometrial cells within 2 min.

Rapid responses to estrogen have also been found in maturing human oocytes and granulosa cells (Morley et al., 1992; Tesarik and Mendoza, 1995). Here, an immediate increase in $[\text{Ca}^{2+}]_i$ was demonstrated after addition of only 0.1 nM 17β -estradiol. These effects are specific for estrogens and related compounds such as estrone, 17α -estradiol, and estriol, whereas progesterone, pregnenolone, testosterone, androstenedione, or 5α -dihydrotestosterone are ineffective. In contrast to findings in other tissues, the estrogen-induced $[\text{Ca}^{2+}]_i$ increase was not affected by the ER agonist/antagonist tamoxifen or by the RNA and protein synthesis blockers actinomycin D or cycloheximide (Revelli et al., 1998). The estrogen-induced $[\text{Ca}^{2+}]_i$ increase in granulosa cells is abolished by pretreatment with inhibitors of inositol phospholipid hydrolysis, such as neomycin and U-73,122 (Shears, 1991). In human oocytes at the germinal vesicle stage, 17β -estradiol induces an influx of Ca^{2+} within seconds (Tesarik and Mendoza, 1995). 17β -Estradiol apparently does not influence the progression of oocytes through meiotic maturation, but it improves the fertilization potential of the in vitro matured oocytes (Tesarik and Mendoza, 1995). In spermatozoa, protein de novo synthesis is very limited and occurs only in mitochondria. Thus, effects induced by estrogen are almost necessarily mediated by nongenomic actions. Early studies point to an enhancement of sperm motility by 17β -estradiol (Beck et al., 1976; Cheng and Boettcher, 1979). Furthermore, oxygen uptake, lactate production, and metabolism of several substrates are increased (Hicks et al., 1972; Hyne et al., 1978). In spermatozoa of fertile and infertile men, addition of 17β -estradiol is claimed to improve the results of the zona-free hamster ova penetration test (Chan et al., 1983). The effects are probably mediated by specific, low-affinity binding sites for 17β -estradiol (Hyne and Boettcher, 1977). Their binding of 17β -estradiol can be reduced by incubation with steroids like progestagens (Hyne and Boettcher, 1978). The receptor is obviously localized in the plasma membrane because specific binding sites for 17β -estradiol could not be detected in the sperm cytosol or sperm nuclei (Cheng et al., 1981). The impact of estrogens on androgen synthesis was the focus of a recently published investigation

by Loomis and colleagues. They found that estrogens ranging from 37 nM to 37 μmol caused decreases in gonadotropin-stimulated 11-ketotestosterone synthesis in testes of a vertebrate species. These effects, which occurred within 5 min, were specific for estrogens (Loomis and Thomas, 2000).

Another focus of nongenomic estrogen effects is the impact on vascular tissues. In human and pig coronary arteries and preparations of rat aortae, ethinyl estradiol or 17β -estradiol elicit an acute and clinically relevant relaxation (Reis et al., 1994; Salas et al., 1994; Yue et al., 1995), which has been suggested to provide a mechanism to explain cardioprotective effects of estrogens in postmenopausal hormone replacement therapy. Moreover, 17β -estradiol leads to an acute relaxation of rat arteries from females, but not males (McNeill et al., 1996). The underlying mechanism of this gender-related difference has still to be settled. Direct Ca^{2+} -antagonistic effects of estrogens on VSMC seem to be involved, whereas the endothelium dependence of relaxation still remains controversial. Farhat et al. (1996) demonstrated that 17β -estradiol increases basal intracellular cAMP in pulmonary VSMC within 5 min. This increase is probably caused by an estrogen-induced activation of membrane adenylate cyclase. Moreover, in PC12 cell membranes, Chen et al. (1998) showed that the increased intracellular cAMP concentration is paralleled by enhanced activation of membrane-bound guanylate cyclase and also of the atrial natriuretic factor-stimulated guanylate cyclase activity. In PC12 cells, catecholamine secretion via membrane depolarization is mediated by Ca^{2+} influx through L- and N-type voltage-sensitive Ca^{2+} channels and P2X_2 receptors. Recently, Kim et al. (2000) reported that 17β -estradiol rapidly reduces the catecholamine secretion by inhibiting these L- and N-type Ca^{2+} channels and P2X_2 receptors. In most of the above-mentioned in vitro and in vivo studies, micromolar concentrations of 17β -estradiol have been applied which exceed plasma levels of estrogens both in physiological conditions and hormone replacement therapy. Since these actions should be assumed as nongenomic steroid effects probably of a nonspecific nature, the clinical relevance of these effects must be discussed cautiously.

Rapid stimulation of eNOS within 5 min induced by 17β -estradiol has been shown in isolated early passage ovine fetal pulmonary artery endothelial cells (Lantin-Hermoso et al., 1997). The effects of estrogens on eNOS have been already described under *Section III.B.1*.

Within the wide scope of nongenomic effects of estrogens described in this section is their impact on intestinal and endocrinological cells. Picotto et al. (1996) have shown that 17β -estradiol induces rapid, direct, and specific effects in isolated duodenal cells resulting in the activation of the cAMP pathway and Ca^{2+} influx. The PLC pathway seems to be involved in the nongenomic regulation of duodenal cell Ca^{2+} concentration since

PLC inhibitors like neomycin completely suppressed 17β -estradiol-induced Ca^{2+} uptake (Picotto et al., 1999).

Estradiol has been shown to increase the pancreatic insulin secretion and to decrease the insulin resistance antagonizing the effects of menopause on glucose and insulin metabolism (Brussaard et al., 1997). Moreover, estrogen prevents male and ovariectomized mice from developing diabetes mellitus (Efrat, 1991). Observations of a recent study show that nongenomic effects are also involved. At physiological concentrations, 17β -estradiol closes rapidly and reversibly K_{ATP} from pancreatic β -cells and depolarizes the plasma membrane, resulting in increased $[\text{Ca}^{2+}]_i$, which in turn enhances the insulin secretion (Nadal et al., 1998). The fast time course and effects of BSA-coupled estrogen on Ca^{2+} signals and K_{ATP} channel activity indicate the nongenomic nature of this effect.

b. Estrogen Receptors for Rapid Signaling. As already mentioned under Section III.B.1., membrane-binding sites for estrogen have been linked to the classic ER, but some studies point to binding sites that could be different from $\text{ER}\alpha$. Estrogen-binding sites in plasma membranes could be found in various tissues like myometrium, liver, and a breast cancer cell line (MCF-7) (Pietras and Szego, 1977; Berthois et al., 1986). Similar results have been reported about the linking of estradiol and 2-OH-estradiol to pituitary plasma membranes (Schaeffer et al., 1980; Bression et al., 1986) or dentritic hypothalamic membranes from guinea pigs (Blaustein et al., 1992). Furthermore, Fiorelli et al. (1996) demonstrated that BSA-coupled estrogen conjugate accumulates on the cell surface of osteoblast- and osteoclast-like cells. Using different BSA-coupled conjugates, researchers have suggested membrane-binding sites for 17β -estradiol in cultured neostriatal neurons (Mermelstein et al., 1996) and in rat brain (Ramirez et al., 1996).

Within 5 to 15 min, 17β -estradiol leads to a rapid and sustained (2 h) tyrosine phosphorylation and activates MAP kinases, ERK1, and ERK2 in PC12 cells which can be blocked by the MAP kinase/ERK kinase1 inhibitor PD98059, but not by the classic ER antagonist ICI-182,780. In $\text{ER}\alpha$ knockout mice, it was demonstrated that the ability of estradiol to phosphorylate ERK persists (Toran-Allerand et al., 1999). Because a new ER gene ($\text{ER}\beta$) was cloned recently (Mosselman et al., 1996), it is not clear whether the effects of estrogen on ERK activation are caused by the latter receptor or a yet unidentified estrogen receptor, such as a putative membrane-associated estrogen receptor.

In summary, 17β -estradiol exerts its nongenomic effects in various organ systems, including the brain and the reproductive system. Therefore, this group of steroids provides the largest array of rapid effects. The rapid actions in the reproductive system may improve the fertilization potential; thus, their further examination is worthwhile. Nongenomic, 17β -estradiol-induced effects are—at least in most cases—likely to be mediated

by a membrane form of ER that is structurally similar if not identical to the classic ER. However, as the spectrum of nongenomic actions is so diverse, more than one mechanism of action has to be assumed, and more than one membrane receptor is likely to be involved.

3. Androgens.

a. Rapid Effects of Androgens. Similar to other steroids, rapid effects of androgens on $[\text{Ca}^{2+}]_i$ have been described in several cells. In human granulosa, luteinizing cells with the addition of 0.1 nM to 1 μM androstenedione (the main intrafollicular androgen) increased $[\text{Ca}^{2+}]_i$ levels within 2 to 5 s, an effect resulting from both transmembrane influx and mobilization of Ca^{2+} from endoplasmic reticulum (Machelon et al., 1998). Treatment with pertussis toxin and the specific PLC inhibitor U-73,122 abolished the effect, suggesting that the intracellular mobilization of Ca^{2+} involves PLC activation and pertussis toxin-sensitive G-proteins. In this context it is noteworthy that the addition of similar concentrations of testosterone did not result in an increase of $[\text{Ca}^{2+}]_i$ in these cells (Machelon et al., 1998).

In contrast, in male rat osteoblasts testosterone has been shown to stimulate $[\text{Ca}^{2+}]_i$ with similar characteristics as described above for androstenedione. In addition, IP_3 and DAG formation was induced within 10 s after addition of testosterone (10 pM–10 nM) (Lieberherr and Grosse, 1994). Interestingly, similar concentrations of testosterone did not elicit an increase in $[\text{Ca}^{2+}]_i$ in female rat osteoblasts, whereas 17β -estradiol increases $[\text{Ca}^{2+}]_i$ at concentrations as low as 1 pM (Lieberherr et al., 1993), an effect not seen in male osteoblasts. This suggests that the rapid effects of androgens and estrogens in rat osteoblasts are sex-dependent (Lieberherr and Grosse, 1994).

Testosterone raises $[\text{Ca}^{2+}]_i$ in activated T cells within seconds at physiological concentrations (1–10 nM). However, the mechanisms underlying this effect are not identical to those described above because the testosterone-induced increase of $[\text{Ca}^{2+}]_i$ results solely from Ca^{2+} influx, which is blocked by the specific Ca^{2+} channel blocker Ni^{2+} (Benten et al., 1997, 1999a). In contrast, the testosterone-induced $[\text{Ca}^{2+}]_i$ increase in mouse IC-21 macrophages (lacking the classic AR) was found to be predominantly due to release of Ca^{2+} from intracellular stores (Benten et al., 1999b). All effects on $[\text{Ca}^{2+}]_i$ described above are independent of genomic actions because they are not inhibited by nuclear AR antagonists such as hydroxyflutamide or cyproterone.

In contrast, a rapid (20–40 s), hydroxyflutamide-sensitive increase of $[\text{Ca}^{2+}]_i$ after testosterone application, although at higher concentrations (0.3–3 μM), has been described in freshly isolated Sertoli cells (Gorczyńska and Handelsman, 1995). Similarly, in human prostate cancer cells (LNCaP cells) mibolerone (dimethylnortestosterone) or 5α -dihydrotestosterone increase $[\text{Ca}^{2+}]_i$ as early as 2 min after treatment by triggering Ca^{2+} influx (Steinsapir et al., 1991). The effect was blocked by pre-

incubation with hydroxyflutamide, suggesting an involvement of the AR and—at least partially—a 5α -reduction of the steroid.

Except for the latter study, in all studies described above, the plasma membrane-impermeable testosterone-BSA conjugate—although at higher concentrations—was able to increase $[Ca^{2+}]_i$, suggesting that testosterone binds to surface receptors.

Rapid androgen action has also been described in the brain. In adult male rats, electrophoretic application of testosterone to individual neurons in the anterior hypothalamus results in an increase in their firing rates within 2 to 30 s (Yamada, 1979). In the lateral hypothalamus of male rats, testosterone increased the spike frequency of neurons within seconds (Orsini et al., 1985). Testosterone metabolites such as androsterone and androstenediol were found to suppress brain activity in the cat within 1 min of i.v. injection (Kubli-Garfias et al., 1982), whereas testosterone and its 5α -metabolites (5α -dihydrotestosterone and 5α -androstenedione) had no effect. Although the involvement of the classic AR was not tested in these studies, the rapid time course suggests a nongenomic effect.

In isolated and perfused rat heart, testosterone (0.1–100 nM) rapidly blocked the adenosine vasodilator effect and increased vascular resistance (Ceballos et al., 1999). In contrast, acute testosterone administration (0.1–100 nM) induced a vasodilation in canine coronary conductance and resistance arteries in vivo (Chou et al., 1996). Direct vasorelaxing effects of testosterone on rat thoracic aorta have been described that are independent of gender and the classic AR (Costarella et al., 1996). However, these effects were found at very high concentrations ranging from 25 to 300 μ M. Therefore, their specificity is questionable. Similarly, high testosterone concentrations have been used in a recent study in which the androgen 5β -dihydrotestosterone has been found to produce relaxation of rat aorta rings precontracted by noradrenaline or K^+ (Perusquia and Villalon, 1999).

Various other nongenomic effects of androgens have been described, including the dihydrotestosterone-induced increase of intracellular cAMP in the human prostate cancer cell line ALVA-41 through the intermediacy of sex hormone-binding globulin (Nakhla and Rosner, 1996) and an interruption of the cell-to-cell communication through gap junction channels by testosterone propionate (1–10 μ M) (Pluciennik et al., 1996). Pretreatment with cyproterone did not prevent the uncoupling action. In addition, testosterone (100 nM), such as progesterone and 17β -estradiol, was also found to depress GnRH receptor-mediated stimulation of low K_m GTPase activity in anterior pituitary membranes from male rats (Ravindra and Aronstam, 1992). Recently, it has been shown that the androgen R1881 stimulates the activity of the extracellular signal-regulated kinase (ERK) in human PMC42 breast cancer cells. This activation oc-

curs promptly after addition of the steroid (1 nM) and is not inhibited by flutamide (Zhu et al., 1999). Similarly, dihydroxytestosterone rapidly increased ERK 1 and ERK 2 phosphorylation in primary prostatic stromal cells. This effect was also found in AR-negative human prostate PC3 cells that were stably transfected with wild-type AR, but it was not found in mock-transfected cells. In both cases the antiandrogen hydroxyflutamide did not inhibit the effects (Migliaccio et al., 1998). Interestingly, several other cells (not specified in the study) did not show the phosphorylation event upon dihydroxytestosterone treatment (Peterziel et al., 1999).

b. Androgen Receptors for Rapid Signaling. Twenty-five years ago, microsomal androgen-binding sites had been described in rat ventral prostate (Robel et al., 1974). Subsequently, using various purification procedures associated with marker enzymes and electron microscopic analyses, the androgen binding activity was found to be selectively associated with fractions rich in rough endoplasmic reticulum and ribosomes (Muldoon et al., 1988; Steinsapir et al., 1990; Steinsapir and Muldoon, 1990, 1991). In rat brain crude synaptosomal membrane preparations, a specific testosterone-binding site was discovered through the use of a radioiodinated testosterone-BSA conjugate (Ramirez et al., 1996). This binding site exhibited a K_d of 30 nM and was proteinaceous in nature. After separation by SDS-PAGE and transfer to nitrocellulose membranes, specific proteins with molecular masses of 35 to 24 kDa were labeled with the radioiodinated conjugate. A membrane-associated androgen-binding protein with similar molecular mass (30–35 kDa) and K_d (39 nM) has been described in *Pseudomonas testosteroni* (Francis and Watanabe, 1983).

Recently, binding sites for testosterone on the surface of T cells and IC-21 macrophages have been detected with testosterone-BSA-FITC by the use of confocal laser scanning microscopy (Fig. 4) and flow cytometry (Benten et al., 1999a,b). Binding of the plasma membrane-impermeable testosterone-BSA (100 nM) conjugate rapidly increases $[Ca^{2+}]_i$. Therefore, the testosterone membrane-binding sites are discussed in the context of nongenomic testosterone action on $[Ca^{2+}]_i$ (as described above). In IC-21 macrophages these binding sites are not identical to the classic AR because this receptor is absent in these cells as shown by three different experimental approaches: 1) incubation of IC-21 cells with an anti-AR antibody (directed against the amino acids 2–21 of the protein) did not significantly label the cells; 2) in Western blot analyses using the same antibody, no immunoreactive band was found; and 3) reverse transcriptase-polymerase chain reaction using primers spanning the DNA-binding domain and three different regions from the carboxyl terminus of the AR-cDNA did not reveal the expected PCR products, whereas the correct DNA bands were found in mouse testes RNA which was used as a positive control (Benten et al., 1999b). In T cells, anti-AR

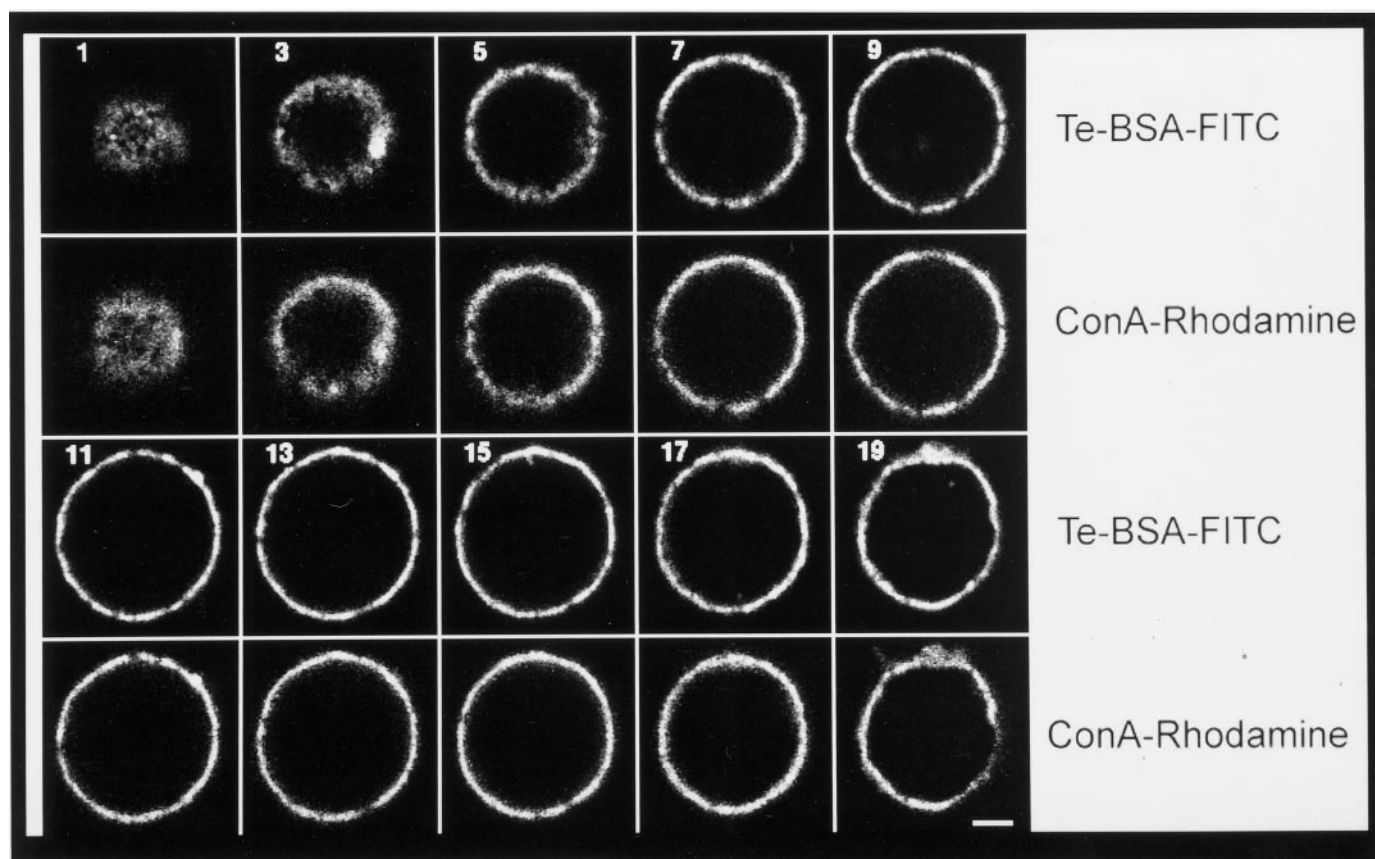


FIG. 4. Confocal optical slices of a T cell incubated with testosterone-BSA-FITC and ConA-Rhodamine. Bar indicates 5 μ M. Reproduced with permission from Benten et al. (1999a).

antibodies directed against the amino or carboxyl terminus of AR did not reveal any significant fluorescence on the cell-surface. Interestingly, despite the common opinion considering T cells to be devoid of AR (Olsen and Kovacs, 1996), the protein was visualized in the cytoplasm of T cells but seems to be inactive in the genomic pathway (Benten et al., 1999a). As already mentioned above, the AR was linked to rapid phosphorylation events in cells of prostatic origin (Peterziel et al., 1999).

Moreover, it has been shown that, in addition to its function as a regulator of free steroid hormone concentrations, sex hormone-binding globulin (SHBG) plays a role in permitting certain steroids to act without entering the cell (Rosner et al., 1999). Upon ligand binding, SHBG interacts with a specific, high-affinity receptor on cell membranes that appears to transduce its signal via a G-protein. The SHBG-receptor complex has been described to cause the activation of adenylyl cyclase and the generation of cAMP within minutes after exposure to the respective steroid, as has been shown in prostate cell membranes, which mediate an increase in intracellular cAMP in response to both androgens and estradiol via binding to a membrane SHBG receptor (Ding et al., 1998; Rosner et al., 1999).

In summary, rapid androgen action was predominantly seen with regard to Ca^{2+} signaling and phosphorylation events. A participation of the AR is likely in some

cases, whereas in others evidence against an involvement of AR exists. The same holds true for inhibitory effects of antiandrogens. The demonstration of the rapid activation of MAP kinase by androgen, which is likely to be mediated by AR, suggests a rather restrictive tissue or organ specificity for this AR response.

B. Glucocorticoids

1. Rapid Effects of Glucocorticoids. Besides long-term effects, glucocorticoids, steroid hormones secreted by the adrenal glands in response to stress situations, rapidly modulate brain function and behavior as well as cellular responses in general (Orchinik et al., 1994; Haller et al., 1998).

Rapid neurophysiological and behavioral effects of corticosterone have been intensively studied in the roughskin newt (*Taricha granulosa*) (Rose and Moore, 1999). In this organism, changes in the electrophysiological excitability and sensory responsiveness of hindbrain neurons were apparent within 3 to 8 min after injection of corticosterone (32 nM) (Rose et al., 1993). Similar rapid effects of systemic glucocorticoid administration on neural activity have been described in the hypothalamus of mammals including rat, cat, and rabbit (Feldman and Dafny, 1970; Filaretov, 1976; Avanzino et al., 1984). The modulation of neurotransmission in the hippocampus by glucocorticoids was investigated in rats:

intraperitoneal injection of corticosterone (2.5 mg/kg) induced a rapid (within 15 min) and transient increase in the extracellular levels of the excitatory amino acids aspartate and glutamate that was not affected by classic antagonists of GR (RU-38486) or MR (RU-28318) or protein synthesis inhibitors (Venero and Borrell, 1999). In rat cerebral cortex synaptosomes and human neuroblastoma cells, the addition of several glucocorticoids including corticosterone and dexamethasone 21-phosphate at a concentration of 1 μ M rapidly (4 min and 15 min, respectively) stimulated the Na^+ -dependent uptake of glutamate (Zhu et al., 1998). Corticosterone was also found to rapidly inhibit arginine vasopressin release in hypothalamic slices of rats at the smallest dose of 100 nM (Liu et al., 1995). Because arginine vasopressin plays an important role in the regulation of hypothalamo-pituitary-adrenal axis activity, it is suggested that rapid glucocorticoid actions may be involved herein.

Rapid effects of corticosterone in the rostral ventrolateral medulla, especially on barosensitive cardiovascular and bulbospinal presympathetic neurons, have been described that result in a very rapid (within 45 s) change in the firing rate. These effects are likely to be nongenomic ones because of the rapid onset of the responses, but they are attenuated by the classic corticosteroid receptor antagonist RU-38486 (Rong et al., 1999). Corticosterone was found to rapidly affect $^{45}\text{Ca}^{2+}$ uptake upon depolarization by high K^+ in synaptic plasma membranes as well as to modulate calmodulin binding, with the maximal effect occurring at a concentration of 1 μ M (Sze and Iqbal, 1994).

The cellular effects described above are mirrored by behavioral changes in several species, elicited a few minutes after corticosterone administration. Recently, it has been shown that corticosterone injections increase the locomotor responses displayed by rats in a novel environment within 7.5 min of systemic administration (Sandi et al., 1996b). The doses used for the experiments (2.5 or 5 mg/kg) are described to mimic plasma concentrations produced by substantial stress (Stein-Behrens et al., 1994). This rapid effect was not blocked by cycloheximide or antagonists for intracellular receptors and seems to involve NO (Sandi et al., 1996a). In white-crowned sparrows it was demonstrated that corticosterone administration caused an increase in perch hopping within 15 min, indicating elevated locomotor activity that is consistent with behavioral responses to natural perturbations (Breuner et al., 1998). In *Taricha*, courtship behavior (clasping of females by males) was significantly suppressed by injection of 2.5 mg/kg corticosterone within 8 min (Orchinik et al., 1991).

In addition, glucocorticoids rapidly modulate cellular responses in general. For example, dexamethasone (0.1 μ M) induces a transient increase in the polymerization of actin within 15 min in human endometrial adenocarcinoma Ishikawa cells (Koukouritaki et al., 1997). Although addition of the glucocorticoid antagonist RU-486

completely abolished this effect, a nontranscriptional pathway seems to be involved, because actinomycin D did not block the dexamethasone-induced actin polymerization (Koukouritaki et al., 1996; Koukouritaki et al., 1997). Recently, acute actions of dexamethasone on nicotine-induced intracellular Ca^{2+} transients, as well as nicotine-induced catecholamine secretion, were examined in porcine adrenal chromaffin cells (Wagner et al., 1999). In the presence of 1 μ M dexamethasone, both the nicotine-induced Ca^{2+} transient and catecholamine secretion were reduced by $\sim 18\%$ within 5 min. Interestingly, in these cells a long-term exposure (48 h) to glucocorticoids leads to the opposite effect, namely, a potentiation of Ca^{2+} signaling (Fuller et al., 1997). Similarly, corticosterone was found to inhibit the nicotine-induced Ca^{2+} influx with a half-maximal inhibitor concentration (IC_{50}) of 0.61 μ M in the pheochromocytoma cell line PC12. Because this effect was mimicked by the PKC activator phorbol 12-myristate 13-acetate, antagonized by PKC inhibitors, and blocked by pertussis toxin, it was concluded that corticosterone acts through the pertussis toxin-sensitive G-protein-PKC pathway (Qiu et al., 1998). In a similar manner, corticosterone inhibited $\Delta[\text{Ca}^{2+}]_i$ induced by high extracellular K^+ in PC12 cells, but with the maximal effect seen at a relatively high concentration of 10 μ M of the steroid (Lou and Chen, 1998). The effects of corticosterone in PC12 cells were also detected in the presence of a corticosterone-BSA conjugate, suggesting that corticosterone might act via membrane receptors.

2. Glucocorticoid Receptors for Rapid Signaling. In the context of the effects described above for *Taricha granulosa*, it is important to note that a membrane receptor for corticosterone has been identified and characterized in this animal (Moore and Orchinik, 1994; Moore et al., 1995). Equilibrium saturation binding experiments with ^3H -labeled corticosterone revealed the presence of high-affinity membrane-binding sites with a K_d of 0.51 nM (Orchinik et al., 1991). The corticosterone membrane-binding protein is enriched in neuronal plasma membrane fractions and responds to nonhydrolyzable guanidine nucleotide analogs in a manner which is typical for G-protein-coupled receptors (Orchinik et al., 1992). The protein was described as an acidic glycoprotein with an apparent molecular mass of 63 kDa (Evans et al., 2000). The membrane protein was found to be highly specific for corticosterone and cortisol. Steroids with high affinity for GR, such as aldosterone and dexamethasone, do not display high affinity for the membrane-associated corticosterone-binding site (Orchinik et al., 1991). Because all of their characteristics are inconsistent with the characteristics of GR, these two receptor proteins appear to be distinct (Evans et al., 2000). In addition, a strong correlation between the pharmacology of the membrane-associated corticosterone-binding site and the potencies of various corticosteroids to inhibit courtship behavior was found, support-

ing the assumption that this protein is involved in the rapid behavioral responses to corticosterone (Orchinik et al., 1991; Moore and Evans, 1999).

Besides this putative distinct amphibian glucocorticoid membrane receptor, it was postulated that a subclass of the GR is localized in the plasma membrane (Gametchu et al., 1999). By the use of an antibody against GR, a GR-like antigen was detected in membranes of S-49 mouse lymphoma cells (Gametchu, 1987; Gametchu et al., 1991) and rat liver (Grote et al., 1993). This finding enabled the development of membrane glucocorticoid receptor (mGR)-deficient and mGR-enriched S-49 mouse lymphoma cells and human CCRF-CEM acute lymphoblastic leukemia cells (Gametchu et al., 1991; Sackey et al., 1997). Studies using these cells revealed some differences between mGR and GR with regard to cellular localization, molecular size, and a slightly different steroid specificity in glucocorticoid binding competition. Scatchard analysis for dexamethasone revealed a K_d value of 239 nM for mGR, compared to a K_d of 19.5 nM for GR (Powell et al., 1999). On the other hand, similarities between both proteins were found. For example, both mGR and GR exhibit epitope recognition for three anti-GR antibodies, bind the same class of steroids, bind GRE DNA and the same heat-shock proteins, and can be phosphorylated (Gametchu et al., 1999). Therefore, it was suggested that mGR is a modified form of GR. Membrane receptor-mediated mechanisms may be related to glucocorticoid-induced cell lysis of lymphoma cells. In this context, a correlation between presence and quantity of mGR and the ability of glucocorticoids to induce lymphocytolysis has been found (Gametchu et al., 1999).

In addition to the membrane receptors mentioned above, membrane-binding sites for several glucocorticoids have been described in various other tissues or cells. Cortisol plasma membrane-binding proteins from chicken-, mouse- and rat liver have been found (Trueba et al., 1987, 1989a; Ibarrola et al., 1996). Subsequently, two types of binding sites have been characterized with K_d values for cortisol of 12 and 344 nM in rat liver. Further purification of the high-affinity binding site revealed two proteins with molecular masses of 52 and 57 kDa exhibiting binding affinities distinct from those of GR. In addition, an antibody against the corticoid-binding globulin failed to detect the cortisol binding site by Western blot analyses. Moreover, membrane-binding sites for corticosterone have been detected in mouse- and rat liver, rat kidney, rat brain, and calf adrenal cortex (Trueba et al., 1989b, 1991; Ibarrola et al., 1991; Guo et al., 1995; Andres et al., 1997). Similar to the above-mentioned cortisol membrane-binding protein from rat liver, the protein from calf adrenal cortex exhibits different binding characteristics compared with the intracellular steroid receptors and the corticoid-binding globulin.

Moreover, another glucocorticoid-binding site has been described in highly purified rat liver plasma membranes (Lackner et al., 1998). This site has been suggested to mediate active transmembrane transport of corticosterone. It does not belong to the ATP-binding cassette/multidrug resistance transporter superfamily and is also distinct from GR (Allera and Wildt, 1992).

In summary, nongenomic action of glucocorticoids mainly affecting neurophysiological and behavioral mechanisms has been described in amphibians, aves, and mammals. The nature of glucocorticoid membrane receptors is still a matter of debate. In consideration of major data in this field, two principle candidates are discussed. On the one hand, membrane glucocorticoid receptors in amphibian brain have been intensively studied that seem to be distinct from GR. On the other hand, data from mouse lymphoma cells suggest that membrane glucocorticoid receptors are a modified form of GR.

C. Mineralocorticoids

1. Rapid Effects of Mineralocorticoids. In addition to the early studies of Spach and Streeten (1964) mentioned above, Moura and Worcel (1984) have reported an early ouabain-independent ^{22}Na efflux by aldosterone in rat tail artery occurring within 15 min and a late Na^+ , K^+ -ATPase-dependent ^{22}Na efflux, which presumably is mediated by genomic mechanisms. Because the initial actions were not inhibited by actinomycin D, nongenomic actions of aldosterone are likely to be involved.

Those studies have suggested that aldosterone may rapidly modulate ion fluxes of different cell types. Indeed, our laboratory was able to demonstrate in the late 1980s that the mineralocorticoid aldosterone significantly increases intracellular Na^+ , K^+ , and Ca^{2+} concentrations in human mononuclear leukocytes (HML). Half-maximal effects have been seen at a concentration of only ~ 0.1 nM (Wehling et al., 1987, 1989, 1990). Although these effects may be mediated by MRs, which have been characterized earlier in HML, the aldosterone concentrations at which half-maximal effects have been observed are significantly lower as are binding characteristics observed in HML (Armanini et al., 1985). Changes of intracellular electrolyte concentrations are accompanied by an increase of cell volume in HML (Wehling et al., 1989) and in endothelial cells (Schneider et al., 1997c), suggesting that those rapid aldosterone effects are involved in the cell volume regulation.

The changes of volume and electrolyte balance are probably induced by a rapid activation of the Na^+/H^+ exchanger, a membrane-bound, electroneutral ion transporter, as the primary step. In HML as well as in vascular smooth muscle cells (VSMC) derived from rat aorta, aldosterone-induced activation of the Na^+/H^+ exchanger occurs within minutes and, thus, is presumably nongenomic in nature. In HML, half-maximal effects for the activation of the Na^+/H^+ exchanger have been seen

at ~ 0.05 nM aldosterone (Wehling et al., 1991b). Similarly, an apparent EC_{50} of ~ 0.2 nM was found in VSMC (Christ et al., 1995a), suggesting that these effects may occur at physiological plasma concentrations of free aldosterone as demonstrated in rats [~ 0.2 nM (Menachery et al., 1991)] and man [~ 0.1 nM (Al-Dujaili and Edwards, 1978)]. Unlike their high affinity to intracellular type I MRs, cortisol and other glucocorticoids were active at supramicromolar concentrations only. Moreover, the type I MR antagonist canrenone did not inhibit these effects. In VSMC, the synthetic steroid fludrocortisone, which is clinically used in an oral formulation for aldosterone substitution in mineralocorticoid deficiency, showed a mineralocorticoid potency similar to that of aldosterone. Rapid activation of the Na^+/H^+ antiporter by aldosterone seemed to be followed by genomic activation of this electroneutral ion transporter. The rapid activation of the antiporter was not blocked by inhibitors of transcription and protein synthesis, whereas effects after 24 h were suppressed (Ebata et al., 1999).

Furthermore, stimulation of the Na^+/H^+ antiporter results in prolonged cellular alkalization of VSMC as demonstrated by BCECF-spectrofluorometry (Wehling et al., 1996). After addition of 100 nM aldosterone, initial acidification is followed by significant alkalization occurring within 2 min, whereas 1 μ M cortisol does not affect intracellular pH. The latter findings are confirmed in a recent paper by Alzamora et al. (2000) Aldosterone induces an increase of Na^+/H^+ -exchanger activity in human strips of uterine and chorionic arteries within a few seconds, which was not affected by spironolactone (10 μ M). However, those effects were blocked by the open-ring MR antagonist RU-28318, a finding which will be discussed in detail below (see *Section IV.C.1.*).

Immediate effects of aldosterone on intracellular cell signaling have also been thoroughly characterized in Madin-Darby canine kidney cells (Gekle et al., 1996). Aldosterone rapidly influences plasma membrane H^+ conductance that in turn increases the activity of the Na^+/H^+ exchanger (Gekle et al., 1997). These results are in line with our findings and with earlier results by the same group (Oberleithner et al., 1987), who demonstrated rapid aldosterone-induced changes in intracellular pH in the distal tubule of the toad kidney. However, the onset of pH modulation was delayed to 15 to 20 min, a time frame that is still compatible with a genomic event. Because it was inhibited by amiloride, the effect was believed to reflect an increased activity of the Na^+/H^+ exchanger.

Rapid effects of aldosterone on Na^+/H^+ -antiporter activity have also been reported in isolated colonic crypts. Aldosterone applied at physiological concentrations (0.08–0.2 nM) increased intracellular pH within 1 min. Ethylisopropylamiloride, and Na^+ -free incubation medium, completely blocked aldosterone-induced effects. As demonstrated in the experiments on Na^+ influx in VSMC (Christ et al., 1995a), these actions are not

blocked by spironolactone, nor by inhibitors of transcription and protein synthesis (Winter et al., 1999). Thus, these findings completely support the findings in kidney cells (Gekle et al., 1996, 1997; Oberleithner et al., 1987) and those of our own group in HML and VSMC (Wehling et al., 1991b, 1996; Christ et al., 1995a,b).

The Na^+/H^+ exchanger is activated by a DAG-dependent mechanism. Therefore, the effects of aldosterone on the products of phosphoinositide hydrolysis were measured in HML and VSMC. In the latter, DAG production was elevated by subnanomolar concentrations of aldosterone, but only by supramicromolar concentrations of cortisol (Christ et al., 1995b). Rapid effects of aldosterone on PKC α have been confirmed in VSMC by immunostaining, which showed translocation of the protein from the cytosol to the plasma membrane (Christ et al., 1995b). Furthermore, rapid activation of the Na^+/H^+ antiporter by aldosterone was blocked by inhibition of protein kinase C activity, again supporting the concept that rapid aldosterone effects are mediated by PKC (Ebata et al., 1999). In contrast to a rapid, DAG-dependent activation of PKC reported by Gekle et al. (1997) and our own group (Christ et al., 1995b), Sato et al. (1997) found a rapid depression of PKC activity by aldosterone in myocyte-enriched cultures from neonatal Sprague-Dawley rat hearts. In addition, Doolan and Harvey (1996a) reported a direct activation of PKC activity by aldosterone in epithelial colon cells, which was obviously not mediated by second messengers. These apparent discrepancies among different investigations with regard to PKC activation cannot be explained at the moment, but may have been generated by the use of different cell types or tissue material.

In addition to DAG formation, intracellular IP_3 levels are increased after aldosterone-induced stimulation of phosphoinositide hydrolysis in HML and VSMC (Christ et al., 1993, 1995a). Aldosterone, as well as fludrocortisone, significantly stimulates the generation of IP_3 within 30 s at an EC_{50} of ~ 0.1 nM. Cortisol is a weak agonist with an EC_{50} of ~ 1 μ M (Christ et al., 1993, 1995a). IP_3 is known to release Ca^{2+} from intracellular stores through IP_3 receptors in the endoplasmic reticulum (Berridge, 1993). Thus, effects of aldosterone on $[Ca^{2+}]_i$ were investigated in VSMC and porcine endothelial cells (Wehling, 1994; Schneider et al., 1997b) by single-cell imaging using the Fura2 method. An almost instant increase of $[Ca^{2+}]_i$ was seen after addition of aldosterone; $[Ca^{2+}]_i$ then leveled off within 2 to 3 min. Consistent with results on Na^+/H^+ antiporter activity and phosphoinositide hydrolysis, half-maximal effects were seen at ~ 0.1 nM aldosterone, whereas cortisol and other glucocorticoids were active at micromolar concentrations only. Pretreatment with spironolactone (10 μ M) for 5 or 30 min did not block the aldosterone-induced increase of $[Ca^{2+}]_i$ (Wehling et al., 1994, 1995). Comparable results on $[Ca^{2+}]_i$ signaling were obtained by Doolan and Harvey (1996a,b) in rat distal colon cells and

T84 colonic cells where aldosterone induced a rapid increase in $[Ca^{2+}]_i$ within ~ 2 min. The authors concluded that aldosterone-induced effects on $[Ca^{2+}]_i$ result from the activation of a Ca^{2+} -influx pathway because no increase in $[Ca^{2+}]_i$ was observed in Ca^{2+} -free incubation medium, whereas the Ca^{2+} response to aldosterone was not reduced after pre-emptying of intracellular Ca^{2+} stores by thapsigargin. Similarly, removal of external Ca^{2+} completely inhibits rapid aldosterone action on $[Ca^{2+}]_i$ in porcine endothelial cells (Wehling et al., 1994; Schneider et al., 1997b). Stimulation of cells in Ca^{2+} -free medium attenuates the Ca^{2+} plateau in VSMC, whereas the initial rise of $[Ca^{2+}]_i$ is still visible (Wehling et al., 1994, 1995). Here, thapsigargin completely blocks aldosterone-induced effects on $[Ca^{2+}]_i$. Thus, in VSMC the sequence of these nongenomic aldosterone effects apparently includes an initial release of $[Ca^{2+}]_i$, followed by a secondary influx of extracellular Ca^{2+} .

In contrast, Ca^{2+} current was significantly increased by long-term incubation but not during short-term incubation (up to 6 h) of aldosterone (1 μM) in isolated adult rat cardiomyocytes (Benitah and Vassort, 1999). In addition, kinetics and affinity of membrane-bound Ca^{2+} channels were not modulated by short-term and long-term application of aldosterone (Benitah and Vassort, 1999). This shows that nongenomic aldosterone effects are restricted to specific target tissues and are not a general housekeeping principle present in all cells or tissues.

In addition to above-mentioned effects, aldosterone elicits a ~ 2 -fold increase of intracellular cAMP levels ($EC_{50} \sim 0.01$ – 0.1 nM) in VSMC within 1 min (Fig. 5), which was found to be Ca^{2+} -dependent. Again, hydro-

cortisone acts only at supraphysiological concentrations (10 μM), and inhibitors like spironolactone, actinomycin D, or cycloheximide had no effects on this feature (Christ et al., 1999a). Because aldosterone induces a time-dependent phosphorylation of CREB, which is involved in the regulation of nuclear transcription, it may be speculated that transcription is indirectly modulated by nongenomic aldosterone signaling.

In contrast to aldosterone-induced effects on intracellular cAMP, 17β -estradiol elevates cAMP at micromolar concentrations only, as has been reported for VSMC of the pulmonary artery (Farhat et al., 1996). This is consistent with the conclusion that aldosterone, not estrogen, is the physiological stimulus for rapid, steroid-induced cAMP signaling in VSMC.

Because these rapid actions are specific for aldosterone, it is tempting to speculate that modulation of intracellular signaling by nongenomic aldosterone actions influences genomic effects of mineralocorticoids via an intracellular cross-talk. This cross-talk may contribute to specific genomic actions of aldosterone (for review see Christ et al., 1999b). Our hypothesis is supported by findings that increases of intracellular second messengers influence genomic steroid actions (Nordeen et al., 1994; Lim-Tio et al., 1997). Furthermore, genomic mineralocorticoid action is modulated by PKA activation (Massaad et al., 1999), which ATP-dependently phosphorylates CREB. Because activating transcription factor/CREB sites have been identified in the promoter regions of putative, aldosterone-regulated proteins (Ahmad and Medford, 1995), increased pCREB may be involved in conferring specificity of aldosterone action at the transcriptional level by nongenomic modulation of intracellular signaling pathways.

2. Mineralocorticoid Receptors for Rapid Signaling. In HML and pig kidney, radioligand binding studies suggest the presence of specific aldosterone membrane-binding sites (Wehling et al., 1991a; Christ et al., 1994; Meyer et al., 1995). These sites exhibit binding characteristics that are different from those of the cloned type I MR (Arriza et al., 1987), but which are in line with those of rapid aldosterone effects. Specific saturable binding of aldosterone to microsomal membranes of HML and kidney was demonstrated with a K_d of ~ 0.1 nM for the radioligand; displacement experiments showed a K_d of ~ 0.1 nM for aldosterone (Wehling et al., 1992a; Christ et al., 1994). Canrenone and cortisol were inactive as ligands up to micromolar concentrations, whereas fludrocortisone and desoxycorticosterone acetate showed intermediate activity. SDS-PAGE done with membrane preparations derived from HML that were photolabeled with a ^{125}I -aldosterone derivative demonstrated significant aldosterone binding by a protein with a molecular mass of ~ 50 kDa which was aldosterone-sensitive, but cortisol-insensitive (Eisen et al., 1996). Using $[^3H]$ aldosterone, specific binding sites were identified and characterized in porcine liver microsomes

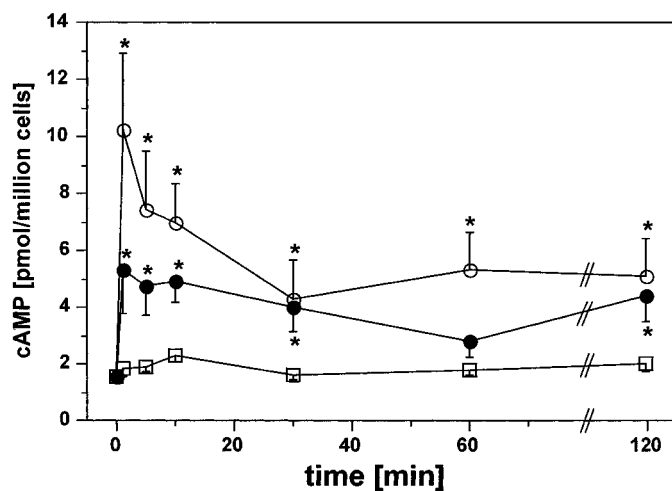


FIG. 5. Time course of intracellular cAMP stimulation in vascular smooth muscle cells by aldosterone and isoproterenol. Cells were incubated in HEPES-buffered, physiological salt solution containing 500 μM IBM-X [control: 0.01% ethanol; □], with aldosterone [100 μM ; ●], or with isoproterenol [10 nM; ○]. Levels of intracellular cAMP were measured by a commercially available specific radioimmunoassay. Values are means \pm S.E.; $n = 5$ (isoproterenol) and $n = 8$ (control, aldosterone); *, $P < .05$ versus control. Reproduced with permission from Christ et al. (1999a).

(Meyer et al., 1995). Maximum binding capacity is ~ 700 fmol mg^{-1} of microsomal protein. Binding of [^3H]aldosterone was saturable, and Scatchard analysis revealed two apparent dissociation constants: $K_{d1} = 11$ nM and $K_{d2} = 118$ nM. The latter binding site most likely reflects a nonspecific interaction, presumably with lipids. In addition, specific binding to plasma membranes of rat kidneys has been described in earlier reports with K_d values for aldosterone binding of 3 to 13 nM (Ozegovic et al., 1988) and 100 nM (Forte, 1972).

In further experiments we examined if aldosterone membrane-binding sites are modified classic MR or if they unquestionably represent an unrelated receptor protein. Therefore, rapid aldosterone signaling was analyzed in skin fibroblasts of wild-type and MR knockout mice (Haseroth et al., 1999). In cells of both sources, aldosterone rapidly increased $[\text{Ca}^{2+}]_i$ (Fig. 6) and cAMP levels within minutes. In cells from knockout mice, these effects were even larger than in cells of wild-type mice. Furthermore, aldosterone was not able to raise intracellular cAMP and $[\text{Ca}^{2+}]_i$ in cells stably transfected with a functional human type I MR (unpublished results). The results of those studies strongly support the hypothesis that the classic MR is dispensable for rapid aldosterone action.

The rapid actions of aldosterone had been characterized by the inability of MR blockers such as canrenone or spironolactone to abolish rapid, aldosterone-induced effects. Therefore, it was hypothesized that the receptors, involved in rapid aldosterone signaling, may be different from the nuclear steroid hormone receptors (Christ et al., 1999b). The latter hypothesis has been challenged by the recent work of Alzamora et al. (2000). Aldosterone induces an increase of Na^+/H^+ -exchanger activity in human strips of uterine and chorionic arteries, which was insensitive to spironolactone (10 μM), but sensitive to the open-ring MR antagonist RU-28318 (Alzamora et

al., 2000). In contrast to spironolactone, which induces an exclusive nuclear localization of the antagonist-receptor complex (Rupprecht et al., 1993), little is known about the mechanisms involved in MR antagonism by RU-28318. It may be hypothesized that the open-ring structure of RU-28318 enables the compound to inhibit rapid aldosterone signaling. Displaying a different structure, the hypothesized membrane receptor of aldosterone may not only bind aldosterone but also RU-28318, in contrast to other MR antagonists, which only bind to nuclear steroid hormone receptors. RU-28318 appears to be a useful tool to further study rapid, aldosterone-induced signaling.

The observation that RU-28318 blocks rapid signaling does not necessarily imply that rapid effects of aldosterone are mediated by the classic MR, an unlikely assumption given the fact that in MR knockout mice aldosterone is still nongenomically active (Haseroth et al., 1999).

In summary, aldosterone affects various intracellular signaling mechanisms through a nongenomic mechanism. Data obtained from MR knockout mice together with the kinetic and pharmacological properties of both the aldosterone membrane-binding sites and the nongenomic aldosterone actions indicate an involvement of a distinct membrane receptor.

D. Neuroactive Steroids

Various steroids synthesized in the adrenal glands and gonads are capable of modulating neuron excitability in the CNS. In many cases they mediate their effects nongenomically and thus differ from steroid hormone actions that regulate transcription through interactions with intracellular receptors [see reviews on history, biosynthesis, and biological activity (Gee et al., 1995; Lambert et al., 1995; Rupprecht, 1997)]. For these compounds the term "neuroactive steroids" has been coined (Majewska et al., 1986), or "neurosteroids" for those that the brain can synthesize *de novo* (Baulieu, 1991). In addition to endogenous steroids such as pregnenolone sulfate, DHEA-S, estradiol, or progesterone for which neuroactive properties have been described (Paul and Purdy, 1992; Rupprecht, 1997), synthetic steroids have been developed recently that share their endogenous counterparts' characteristic of modulating a variety of G-protein-coupled receptors and ligand-gated ion channels (Gasior et al., 1999). Just as diverse as the array of compounds with neuroactive properties known today are the physiological effects they exert. Neuroactive steroids influence sleep patterns, the reaction to stress, memory function, brain plasticity, vigilance, and mood (Rupprecht and Holsboer, 1999). Hence they may have a therapeutic potential far beyond today's applications.

Cholesterol and the steroids derived are highly lipophilic substances. Due to this characteristic, they readily cross the blood-brain barrier. Therefore, peripherally synthesized steroids have the theoretical poten-

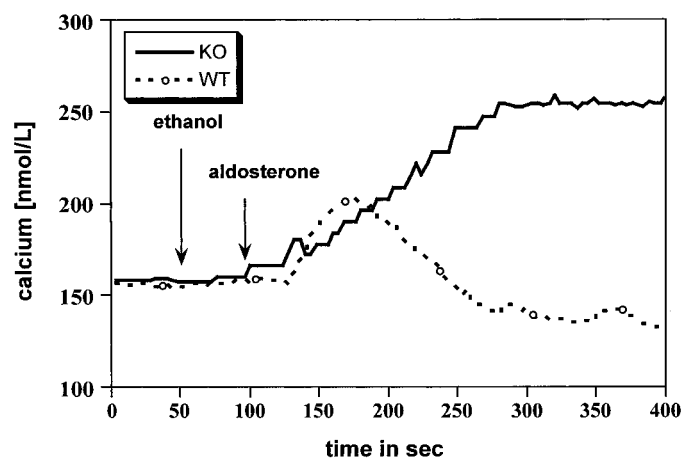


FIG. 6. Representative experiment displaying the time course of $[\text{Ca}^{2+}]_i$ after aldosterone stimulation. Skin cells derived from wild-type mice (WT) or MR knockout mice (KO) were stimulated with 0.01% ethanol (vehicle) and 10 nM aldosterone. Modified with permission from Haseroth et al. (1999).

tial to act within the CNS. In addition, de novo hormone synthesis in the CNS has been demonstrated for the conversion of cholesterol to the neuroactive pregnenolone in the rodent brain (Hu et al., 1987; Cheney et al., 1995) and in nonmammals (Tsutsui and Yamazaki, 1995; Takase et al., 1999). The primary production site of neurosteroids seems to be glial cells (Tsutsui et al., 2000), but de novo neurosteroid production has also been reported for Purkinje cells (Ukena et al., 1999). It remains to be determined if differential production sites for neurosteroids play an important role, if any, in the physiological or pathophysiological effects that neurosteroids convey.

In addition to its ability to synthesize neuroactive steroids de novo, the brain also harbors enzymes to metabolize circulating steroid hormones (Celotti et al., 1992; Mellon, 1994). Pregnenolone, one of the most thoroughly investigated neuroactive steroids, is partly metabolized to DHEA. The conjugated sulfate esters pregnenolone sulfate and DHEA-S can be found in concentrations in the brain that exceed those of the free steroids by far (Baulieu, 1998). In addition, androgens are metabolized in the brain to estrogens and 5α -reduced androgens, such as 5α -androstane- 17β -ol-3-one (dihydrotestosterone) and 5α -androstane- $3\alpha,17\beta$ -diol (Celotti et al., 1992).

However, there are extraordinary species differences that complicate the extrapolation of findings in nonmammals or even rodents to primates and humans in particular. In primates, progesterone is mainly metabolized to 20α -hydroxy-4-pregnen-3-one by 20α -hydroxysteroid oxidoreductase (Tabei and Heinrichs, 1974; Korneyev et al., 1993), a metabolite with a low modulatory potential at the GABA_A receptor in the rat brain due to a lacking 3α -hydroxyl or 20 -ketone group. These groups are responsible for GABA receptor modulation of α -reduced pregnanes (Harrison et al., 1987; Lan et al., 1991). Because most GABA_A receptor-modulating progestins may originate in the peripheral endocrine system, limiting the importance of progesterone metabolism in the rat CNS, it remains to be determined which clinically important function, de novo synthesis of neuroactive hormones has in humans.

A major focus of future investigations should be research on neurosteroid effects in humans. Beyond the scope of this review but certainly worth considering are efforts to use modern imaging techniques to measure the effect of neuroactive steroids in vivo. The Positron Emission Tomography and functional Magnetic Resonance Imaging may offer an approach to evaluate such effects safely and reliably (Resnick et al., 1998; Maki and Resnick, 2000).

1. Rapid Effects of Neuroactive Steroids. Neuroactive steroids mediate their effects via classic genomic mechanisms in analogy to the mechanisms already described in detail (see *Section II.A.*), as well as nongenomically via direct modulation of neurotransmitter

receptors (e.g., GABA_A and NMDA receptors). Because steroids that are capable of modulating GABA_A receptors such as $3\alpha,5\alpha$ -THP or $3\alpha,5\alpha$ -THDOC are unable to bind to PR (Paul and Purdy, 1992), the only way by which these compounds may confer genomically mediated effects is by intracellular metabolites (Rupprecht et al., 1993). In fact, cytoplasmatic oxidation of $3\alpha,5\alpha$ -THP and $3\alpha,5\alpha$ -THDOC to $3\alpha,5\alpha$ -dehydroprogesterone and $3\alpha,5\alpha$ -dehydrodeoxycorticosterone may affect transcription because the latter are ligands of PRs (Rupprecht, 1997). Evidence also derives from a finding that links the enhanced expression of the gene encoding for the $\alpha 4$ subunit of the GABA_A receptor to the development of progesterone withdrawal symptoms after withdrawal of progesterone and thus $3\alpha,5\alpha$ -THP, its metabolite. Blockade of the $\alpha 4$ gene transcript prevents the observed withdrawal symptoms (Smith et al., 1998). $3\alpha,5\alpha$ -THP and $3\alpha,5\alpha$ -THDOC are also involved in the expression of genes encoding for corticotropin-releasing hormone (Patchev et al., 1994) and vasopressin (Patchev et al., 1996). However, difficulties in distinguishing between genomically and nongenomically mediated steroid effects often arise due to a variety of confounding factors. The time frame for nuclear transduction mechanisms and membrane-mediated nongenomic effects is not necessarily mutually exclusive. The latter may occur with a latency of minutes, and very fast genomic responses to steroid treatment, measured as changes in c-myc mRNA levels of chick oviduct, have also been reported after only 10 min following administration of progesterone (Fink et al., 1988). With the aid of specific pharmacologic receptor antagonists to membrane receptors as well as intracellular receptors, studies that aim to exactly distinguish between nongenomic and genomic steroid action can be expected.

Today various neuroactive steroids have been shown to interact with different types of neuron receptors. The swiftness of onset as well as evidence for their independence from the presence of a nuclear apparatus define these effects of steroids on neuronal function as nongenomically mediated. So far the interaction of neuroactive steroids with the GABA_A receptor has been most thoroughly investigated. The most important findings are summarized under *Section III.B.3.*

In addition, allosteric interactions of neuroactive steroids have been reported for numerous other receptors, among them the *N*-methyl-D-aspartate (NMDA) receptor, the 5-HT₃ receptor, and the glycine receptor. These interactions will be briefly recapitulated in the following section. In addition, the evidence for the involvement of G-proteins in steroid modulation of neuronal activity will be reviewed.

2. Neurosteroid Receptors for Rapid Signaling.

a. γ -Aminobutyric Acid_A Receptor. Different neuroactive steroids have been shown to exert extremely diverse psychoneurological effects by interaction with the GABA_A receptor, mainly depending on their specific po-

tential to allosterically enhance or block the action of GABA or other GABAergic substances like barbiturates and benzodiazepines. Among the most potent positive allosteric modulators of GABA_A receptors are 3 α ,5 α -THP and 3 α ,5 α -THDOC. Hence, the effects they confer are similar to those seen after benzodiazepine or barbiturate administration and include sedation, anticonvulsion, antinociception, and anxiolysis (Rupprecht, 1997). As early as 1942, Selye described the anesthetic properties of steroid hormones (Selye, 1942). The clinical use of steroid anesthetics such as alphaxalone, however, has so far been inhibited by an unacceptable frequency and severity of adverse drug reactions (Paul and Purdy, 1992).

Possibly, the anxiolytic properties of neurosteroids are of greater clinical potential. Administration of THP (Bitran et al., 1991; Wieland et al., 1995), THDOC (Crawley et al., 1986), allopregnanolone (Zimmerberg et al., 1994; Vivian et al., 1997), alphaxalone (Britton et al., 1991), or ganaxolone (Beekman et al., 1998) is apparently associated with anxiolysis in various test paradigms. The observed anxiolytic effect was independent of the sedative properties of neuroactive steroids. These findings, however, are based on experiments in rodents. Clinical investigations studying a possible anxiolytic effect of positive allosteric modulators at the GABA_A receptor have not been reported so far.

However, in observational studies it has been shown that in women progesterone is beneficial for the woman's mood and some physical symptoms in patients suffering from premenstrual syndrome (Dennerstein et al., 1985). These effects seem to be mediated via an allosteric modulation of the GABA_A receptor by the progesterone metabolite 5 α -pregnanolone (Vanselow et al., 1996). This study, however, did not differentiate between the pregnenolone stereoisomers. Whether progesterone or its metabolites themselves improve the clinical condition in women with premenstrual syndrome remains uncertain because a relationship between progesterone and other neuroactive steroid levels and clinical symptoms does not seem to exist (Schmidt et al., 1994). Sleeplessness, increased anxiety, and depressive symptoms which can often be found in the premenstrual syndrome are also observed during benzodiazepine withdrawal. Micronized progesterone did not alleviate the symptoms of diazepam withdrawal in patients who had taken diazepam daily for at least 1 year. In animal models of drug dependence, however, allopregnanolone has been effective in reducing anxiety and hyperlocomotion associated with benzodiazepine withdrawal (Reddy and Kulkarni, 1997).

So far no studies in humans have been reported that clearly corroborate a role for neuroactive steroids in drug withdrawal.

The anticonvulsant properties of neuroactive steroids have already been investigated in preclinical as well as clinical settings. In animal models, allopregnanolone

(Belelli et al., 1990; Kokate et al., 1996), pregnanolone (Belelli et al., 1990; Gasior et al., 1997), THDOC (Kokate et al., 1996), and ganaxolone (Carter et al., 1997) have been shown effective in the reduction of seizures (Gasior et al., 1999). Ganaxolone is a synthetic 3 β -methyl-substituted analog of the endogenous neuroactive steroid 3 α -hydroxy-5 α -pregnan-20-one. Ganaxolone inhibits binding of the GABA_A receptor-chloride channel ligand *t*-[³⁵S]butylbicyclo-phosphorothionate and enhances binding of the benzodiazepine site ligand flunitrazepam (Carter et al., 1997). This evidence characterizes ganaxolone as a positive allosteric modulator of GABA at its receptor. In complex partial seizures in adults and refractory seizures in children, ganaxolone has been claimed to be efficacious in antiepileptic therapy (Bialer et al., 1999; Gasior et al., 1999). In women with partial epilepsy, progesterone infusion significantly decreased the spike frequency in simultaneously recorded EEGs (Backstrom et al., 1984). However, this study can only be considered hypothesis-generating rather than testing because of its small sample size of seven women.

More profound clinical experience derives from studies investigating GABA_A receptor-modulating neurosteroids in sleep disorders. Progesterone has been demonstrated to shorten sleep latency and to increase nonrapid eye movement sleep duration (Friess et al., 1997). The hypothesis that allosteric modulation of the GABA_A receptor is involved in sleep disturbances in humans is further backed by the finding that DHEA-S, an allosteric inhibitor of GABA_A receptors, is markedly decreased in patients with chronic fatigue syndrome (Kuratsune et al., 1998). DHEA has also been investigated with respect to its sleep-modulating properties. DHEA administration results in an increase in REM sleep after oral administration while simultaneously enhancing EEG activity in the sigma frequency range during REM sleep. This finding is compatible with a mixed GABA-agonistic/antagonistic profile of DHEA as a modulator of the GABA_A receptor (Friess et al., 1995). In rodents, however, it has recently been demonstrated that the sulfate ester of DHEA administered intraperitoneally did not modulate sleep architecture, although it did affect sleep EEG patterns (Schiffelholz et al., 2000). It may be speculated that the hydrophil ester of a steroid is less likely to cross the blood-brain barrier and therefore is unable to exert an anticipated effect or that interspecies differences account for the seemingly contrasting results. The memory-enhancing effects described for DHEA and its sulfate ester DHEA-S have partly been explained by changes in sleep patterns mediated via the GABA_A receptor. However, a memory-enhancing effect of neuroactive steroids may also be conferred via an allosteric interaction with another receptor of the CNS, the NMDA receptor (see *Section IV.D.2.b.*).

Still, the link between the clearly established structure-activity relationship of neuroactive steroids as al-

losteric modulators of neuron excitability at the GABA_A receptor and observed behavioral effects of these substances remains somewhat elusive.

The specific characterization of steroid effects, attributed to the interaction of various compounds with the GABA_A receptor, has mainly been performed in rodents and in vitro. But epidemiological evidence and preliminary clinical observations suggest a pivotal role for neuroactive steroids in the natural history and possibly in the treatment of many psychiatric and neurologic disorders (Rupprecht, 1997; Herbert, 1998; Herzog, 1999). Further interventional clinical studies will have to be performed to elucidate the function of steroids that allosterically modulate the GABA_A receptor in humans.

b. N-Methyl-D-aspartate Receptor. The NMDA receptor, a member of the glutamate receptor family, has been characterized as sensitive for the modulatory action of pregnenolone sulfate (Wu et al., 1991; Park-Chung et al., 1994), pregnenolone hemisuccinate (Weaver et al., 1997a), and 17 β -estradiol (Weaver et al., 1997b).

It has been reported that pregnenolone hemisuccinate, which is a synthetic homolog of naturally occurring pregnenolone sulfate, inhibits NMDA-induced currents and cell death in primary cultures of rat hippocampal neurons. It reveals sedative, anticonvulsant, and analgesic properties consistent with an action at NMDA-type glutamate receptors in rats. Intravenous administration of pregnenolone hemisuccinate to rats at doses that have no sedating effects, following focal cerebral ischemia, reduces cortical and subcortical infarct size (Weaver et al., 1997a). These supposedly neuroprotective effects are shared by 17 β -estradiol in the animal model. 17 β -Estradiol protects against NMDA-induced neuronal death by directly inhibiting the NMDA receptor (Weaver et al., 1997b). This finding is supported by epidemiological studies, implying that women on estrogen hormone replacement therapy have a decreased risk and lesser severity of dementia (Mortel and Meyer, 1995; Schneider et al., 1997a). In other epidemiological studies, decreased levels of circulating neuroactive steroids have been associated with various forms of dementia (Baulieu, 1998; Hillen et al., 2000). In accordance with these results, pregnenolone has also been suggested to enhance memory performance. This hypothesis is based on findings in mice in which pregnenolone, pregnenolone sulfate, and DHEA caused improvements in footshock active avoidance training (Flood et al., 1992). The memory-enhancing effect of pregnenolone, which has been verified in different test paradigms in rodents (Mathis et al., 1994), has been linked to its allosteric modulation of the NMDA receptor because pregnenolone sulfate reduces scopolamine-induced learning deficits (Meziane et al., 1996). Further support for a nongenomically mediated effect of pregnenolone sulfate at the NMDA receptor derives from the finding that it blocks learning and memory impairment induced by D-2-amino-5-phospho-

novalerate, a competitive NMDA receptor antagonist (Mathis et al., 1996). Pregnenolone sulfate has also been shown to act as a coagonist at the NMDA receptor, allosterically modulating receptor currents within seconds (Bowlby, 1993).

In the light of available mechanistic, functional, and epidemiological evidence the extent remains to be determined to which observed in vivo effects of neuroactive steroids are indeed nongenomically mediated. In addition to NMDA and GABA_A receptors, other receptors may also be involved in conferring nongenomically mediated effects of neuroactive steroids.

c. Sigma1 Receptor. The sigma1 receptor protein has recently been purified, and its cDNA has been cloned in several species (Hanner et al., 1996; Kekuda et al., 1996; Seth et al., 1998). Ligands of the sigma1 receptor exert a potent neuromodulation at the NMDA-receptor complex. In excitatory neurotransmitter systems, including glutamate and cholinergic systems, the sigma1 receptor ligands seem to be potent neuromodulators (Maurice et al., 1999). Consequently, selective sigma1 agonists show neuroprotective properties (Maurice et al., 1997) and beneficial effects in depression (Reddy et al., 1998) and memory processes (Reddy et al., 1998; Urani et al., 1998). The memory enhancing characteristics of DHEA-S and pregnenolone sulfate can be blocked by concurrent administration of a prototype sigma1 receptor antagonist (Reddy et al., 1998). Because neuroactive steroids show the potential to inhibit the binding of sigma1 receptor radioligands in vitro and in vivo, a direct interaction between neuroactive steroids and sigma1 receptors seems to exist (Maurice et al., 1999). These results suggest the involvement of CNS sigma1 receptors in the memory- and behavior-modulating effects of neuroactive steroids.

Progesterone, as an abundantly present neuroactive hormone in many mammalian species, has been shown to be a ligand for the sigma receptor in vitro (McCann and Su, 1991; Meyer et al., 1998b). In rats, progesterone acts as an 'antagonist' at sigma receptors (Maurice et al., 1998). The resulting changes in the function of sigma receptors may partly explain emotional changes occurring in women during pregnancy and postpartum (Bergeron et al., 1999). How far these phenomena are mediated nongenomically or genomically or via a cross-talk involving second messengers, however, has not been established. The time frame of effects within minutes demonstrates the importance of nongenomically mediated effects.

d. 5-Hydroxytryptamine Type 3 Receptor. The gonadal steroids, 17 β -estradiol and progesterone, have been described as functional antagonists at the 5-HT₃ receptor in whole-cell voltage-clamp recordings of cells stably expressing the 5-HT₃ receptor. Functional antagonistic properties at this ligand-gated ion channel could also been shown for 17 α -estradiol, 17 α -ethinyl-17 β -estradiol, testosterone, and allopregnanolone but not for

pregnenolone sulfate and cholesterol (Wetzel et al., 1998). From comparative studies using various alcohols, it is thought that the modulation of 5-HT₃-receptor function by steroids is dependent on their respective molecule structure. The antagonistic action of steroids at the 5-HT₃ receptor is not mediated via the serotonin-binding site. This can be concluded because the steroids did not alter the binding affinity of [³H]GR65630 to the 5-HT₃ receptor, and kinetic experiments revealed a different response pattern to 17 β -estradiol when compared with the competitive antagonist metoclopramide (Wetzel et al., 1998). Gonadal steroids probably interact allosterically with the 5-HT₃ receptor at the receptor-membrane interface as has been shown for progesterone (Wu et al., 2000). Progesterone also inhibited the 5-HT₃ response noncompetitively by a voltage- and agonist-independent mechanism that was distinct from that of open-channel blockers (Wu et al., 2000). Activation of 5-HT₃ receptors is, among others, involved in the development of nausea.

Nausea is a common symptom in pregnancy and is also observed in other psychiatric disorders. Circulating levels of gonadal steroids are altered in these conditions, and therefore nausea may be due to the functional antagonism of gonadal steroids at the 5-HT₃ receptor.

e. Glycine Receptor. The glycine receptors belong to the same superfamily of transmitter-gated ion channels as the GABA_A receptor (Kuhse et al., 1995; Soloviev et al., 1996). The glycine receptor probably consists of two subunits (α and β), and a number of isoforms exist that confer distinct pharmacological and biophysical characteristics upon the receptor (Rundstrom et al., 1994; Kuhse et al., 1995; Wafford et al., 1995). The functional properties of the inhibitory receptors are likely to be heterogeneous and may be brain region- or even neuron-specific (Pistis et al., 1997).

Pregnenolone sulfate rapidly and reversibly exerts its inhibitory effects on the glycine receptor-mediated response to glycine-induced currents in a dose-dependent manner. The fact that antagonism of the glycine response by pregnenolone sulfate is neither voltage- nor agonist-dependent suggests that pregnenolone does not act as an open-channel blocker. Furthermore, inhibition of glycine-induced currents by pregnenolone sulfate appears to be mediated competitively, because pregnenolone sulfate induces a parallel, rightward shift of the glycine dose-response curve (Wu et al., 1997).

In contrast, the neuroactive steroid 5 α -pregnan-3 α -ol-20-one neither activated nor inhibited glycine-invoked currents in *Xenopus* oocytes expressing human recombinant glycine receptors (Pistis et al., 1997). Therefore, unclarified specific structure prerequisites of neuroactive steroids may exist to modulate glycine-receptor activity.

Apart from allosteric modulation of neurotransmitter receptors, attention should also be paid to reports on rapid steroid effects on neuronal activity apparently linked to G-proteins. Neuroactive steroids may activate

G-proteins and G-protein-regulated second messenger systems or ion channels.

Estradiol is one of the steroids that exerts its neuroactive effects at least in part via G-protein-coupled responses. In striatal neurons, estradiol in physiological concentrations can suppress Ca²⁺ channels. Intracellular dialysis with guanosine 5'-O-(3-[³⁵S]-thio)-triphosphate prevents reversal of the modulation, suggesting that 17 β -estradiol acts via G-protein activation. Additional support for the suggestion that the noticed effects are mediated nongenomically results from the observation that the effects occurred within seconds of estradiol administration (Mermelstein et al., 1996). In hippocampal neurons, similar inhibitory effects on Ca²⁺ currents of pregnenolone (Ffrench-Mullen and Spence, 1991; Ffrench-Mullen et al., 1994) and allotetrahydrocorticosterone (Ffrench-Mullen and Spence, 1991) were observed. Estradiol appears to modulate neuron excitability through rapid changes of K⁺ conductance of the postsynaptic membrane in amygdala neurons (Nabekura et al., 1986). This effect may be mediated by cAMP, suggesting a G-protein activation of adenylate cyclase via a putative estradiol receptor.

In summary, neuroactive steroids have the potential to powerfully modulate biophysical processes in the CNS, entailing diverse behavioral and psychopharmacological effects in humans and other species. Steroids allosterically modulate various neurotransmitter receptors. So far, this nongenomic mechanism seems to be the most important one, and it is by far the most thoroughly investigated mechanism by which steroids alter neuronal function. G-protein-coupled effects may present another nongenomic way of action of neuroactive steroids at numerous neurons. In addition, neuroactive steroids have been shown to genomically modulate the GABA_A receptor, and it is likely that structurally related neurotransmitter receptors may also be influenced by neuroactive steroids. Therefore, an intracellular cross-talk between nongenomic and genomic effects of neuroactive steroids may frame the molecular scaffolding for CNS steroid action. The diverse in vivo actions of neuroactive steroids depend on lacking specificity of natural and synthetic steroids to uniquely bind to only one neurotransmitter receptor. The metabolism of neuroactive steroids with metabolites that exhibit different pharmacological profiles compared to their precursors is also responsible for the variety of effects of a single steroid. As yet, no derivatives of naturally occurring or synthetic steroids have been developed that show exclusive receptor specificity. In light of these findings, it may be recognized that a great potential for pharmacological intervention is not due to a specific interaction of a steroid with a neurotransmitter receptor but may rather be an expression of a re-establishment of neuroactive steroid homeostasis in the CNS that is impaired in various physiological as well as pathological clinical conditions. This approach already seems to be a reality in patients

with depressive symptoms in whom a disequilibrium of neuroactive steroids can be corrected by tricyclic antidepressants. In other psychiatric and neurologic disorders, pharmacological intervention should also be probed for possibilities to correct a disequilibrium of neuroactive steroids.

E. Vitamin D₃

1. *Rapid Effects of Vitamin D₃*. 1 α ,25-(OH)₂D₃, the major hormonally active form of vitamin D, has been found to rapidly stimulate a variety of signal transduction systems including Ca²⁺ influx, release of Ca²⁺ from intracellular stores, modulation of adenylate cyclase, PLC and protein kinase activities, and changes in the phosphorylation status of cellular proteins (de Boland and Nemere, 1992).

One of the first nongenomic effects of 1 α ,25-(OH)₂D₃ to be studied in detail was the stimulation of Ca²⁺ transport in the perfused chick duodenum (termed transcalcaltachia) (see Section III.B.2.). Here, the application of 1 α ,25-(OH)₂D₃ at subnanomolar amounts to the basal lateral surface of the intestinal epithelial cell resulted in a very prompt increase in the rate of appearance of ⁴⁵Ca²⁺ in the perfusate exiting via the celiac artery (Norman et al., 1993a).

As stated above (Section III.B.2.), a series of vitamin D₃ analogs have been identified that activate only subsets of the biological responses of the hormone (Norman et al., 1997, 1999). So far, this feature is unique among all other steroid hormones. Several studies clearly show that the structural characteristics of the analogs that activate membrane-initiated, rapid pathways (*cis*-conformation) are distinct from those that bind to the nuclear receptor (Norman et al., 1996) and are only weak agonists for the genomic responses (Zhou et al., 1992; Bouillon et al., 1995). In contrast, translocked analogs did not promote transcalcaltachia even at concentrations up to 6.5 nM, and a further analog, the 1 β -epimer of 1 α ,25-(OH)₂D₃ (1 β ,25-(OH)₂D₃) has been found to potentially antagonize the 1 α ,25-(OH)₂D₃-mediated response (Norman et al., 1993a). In a recent study, addition of two hybrid analogs of 1 α ,25-(OH)₂D₃ that were modified on the A-ring and the C, D-ring side chain were described to have an influence on proliferation rate, proteoglycan production, and PKC activity of rat chondrocytes (Boyan et al., 1997; Greising et al., 1997). However, effective binding of these analogs to the classic VDR was only 0.1% relative to genuine 1 α ,25-(OH)₂D₃.

Rapid actions on Ca²⁺ fluxes in bone-forming osteoblasts which function as an integral part of the vitamin D endocrine system and are main target cells for 1 α ,25-(OH)₂D₃ have been intensively studied in recent years. An increase in [Ca²⁺]_i occurring within minutes in response to treatment with 1 α ,25-(OH)₂D₃ (10–100 pM) has been found in primary cultures of mouse osteoblasts (Lieberherr, 1987). Similar results have been described in rat osteosarcoma cells (ROS 17/2.8) (Caffrey and

Farach-Carson, 1989; Civitelli et al., 1990; Baran et al., 1991). Interestingly, this rapid increase of [Ca²⁺]_i has also been described in the osteoblastic cell line ROS 24/1 which lacks VDR (Baran et al., 1991), indicating the involvement of a signaling pathway different from that of genomic mechanisms. Similar to transcalcaltachia, 1 α ,25-(OH)₂D₃ analogs that stimulate Ca²⁺ influx bind poorly to VDR from osteoblasts (Farach-Carson et al., 1991). Moreover, 1 β ,25-(OH)₂D₃ was active as an antagonist (Norman et al., 1993b). Further studies on the rapid 1 α ,25-(OH)₂D₃-induced increase of [Ca²⁺]_i in osteoblasts or related cell lines showed that this effect involved both release of Ca²⁺ from intracellular stores and extracellular Ca²⁺ influx probably via an L-type voltage-sensitive Ca²⁺ channel (Lieberherr, 1987; Caffrey and Farach-Carson, 1989; Civitelli et al., 1990).

Since most nongenomic actions of 1 α ,25-(OH)₂D₃ and other steroids involve Ca²⁺ as second messenger, a rapid increase in [Ca²⁺]_i following 1 α ,25-(OH)₂D₃ stimulation has been described in several other cells or tissues. For example, in skeletal muscle cells (Vazquez et al., 1999) and in both cardiac muscle tissue and cultured chick cardiac muscle cells, physiologically relevant doses of 1 α ,25-(OH)₂D₃ have been found to induce an increase of [Ca²⁺]_i within minutes, an effect which was independent of RNA or protein synthesis. Similar to osteoblasts, this effect seems to involve a voltage-dependent Ca²⁺ channel (Selles and Boland, 1991; de Boland and Boland, 1994).

In addition to the activation of Ca²⁺ influx, 1 α ,25-(OH)₂D₃ rapidly leads to hydrolysis of membrane lipids to produce second messengers that cause release of Ca²⁺ from intracellular stores, especially the endoplasmic reticulum. In female rat osteoblasts, 1 α ,25-(OH)₂D₃ (100 pM) transiently increases [Ca²⁺]_i within 5 s via IP₃ formation, an effect involving PLC- β (Le Mellay et al., 1997). Moreover, rapid, 1 α ,25-(OH)₂D₃-induced hydrolysis of membrane phosphoinositides generating DAG and IP₃ has been described in rat colonocytes and Caco-2 cells. The response was dose-dependent, with the lowest effective concentration of 100 pM (Wali et al., 1990, 1992). Comparable 1 α ,25-(OH)₂D₃-induced effects on the generation of these compounds have also been found in a variety of other cells, including enterocytes (Bourdeau et al., 1990), dispersed porcine parathyroid cells (Lieberherr, 1987), chick embryo muscle cell cultures (Morelli et al., 1993), osteoblasts (Sorensen and Baran, 1995), ROS 17/2.8 cells (Civitelli et al., 1990), hepatocytes (Baran et al., 1988), and keratinocytes (Tang et al., 1987). In rat skeletal muscle, the involvement of PLC and a pertussis toxin-sensitive G-protein in the rapid 1 α ,25-(OH)₂D₃-induced release of IP₃/inositol phosphates and DAG has been shown (Morelli et al., 1996; Facchinetti et al., 1998). In addition, 1 α ,25-(OH)₂D₃ was found to rapidly activate phospholipase D in Caco-2 cells (Khare et al., 1999), chick myoblasts

(Facchinetti et al., 1998), and rat skeletal muscle (Morelli et al., 1996).

Since DAG can cause the activation of PKC, the influence of $1\alpha,25\text{-(OH)}_2\text{D}_3$ on this protein has also been examined in several tissues or cells. In growth zone chondrocytes, $1\alpha,25\text{-(OH)}_2\text{D}_3$ (10 nM) causes a rapid increase in PKC activity, which was insensitive to actinomycin D and cycloheximide (Sylvia et al., 1996; Boyan et al., 1999). Analogs of $1\alpha,25\text{-(OH)}_2\text{D}_3$, which exhibit <0.1% of the binding to the intracellular receptor found for the secosteroid, elicit comparable increases in PKC-specific activity (Greising et al., 1997). In addition, a rapid activation of PKC by $1\alpha,25\text{-(OH)}_2\text{D}_3$ has been reported in rat epithelium cells (Wali et al., 1990). Also, $1\alpha,25\text{-(OH)}_2\text{D}_3$ has been shown to directly activate PKC, indicating the possibility of a $1\alpha,25\text{-(OH)}_2\text{D}_3$ -binding domain on the enzyme (Slater et al., 1995).

Because PKC activation is linked to the activation of MAP kinase, the influence of $1\alpha,25\text{-(OH)}_2\text{D}_3$ on this signaling protein was analyzed. Recently, it was shown that $1\alpha,25\text{-(OH)}_2\text{D}_3$ is able to stimulate both Raf kinase and MAP kinase pathways in keratinocytes (Marcinkowska et al., 1997; Gniadecki, 1998), chick enterocytes (de Boland and Norman, 1998), and NB4 cells (Song et al., 1998), indicating that the rapid response signal-transduction components of the $1\alpha,25\text{-(OH)}_2\text{D}_3$ may be involved in the regulation of cell growth. In NB4 cells, 6-*s-cis*-locked analogs of $1\alpha,25\text{-(OH)}_2\text{D}_3$ (10 nM) increased MAP kinase phosphorylation with the same efficacy as $1\alpha,25\text{-(OH)}_2\text{D}_3$ itself. The translocked analogs were inactive, and $1\beta,25\text{-(OH)}_2\text{D}_3$ had antagonist-like properties (Song et al., 1998). Because in other systems the MAP kinase pathway has been shown to play a role in transducing the ligand signal from the outer cell membrane to the nucleus, it was postulated that the rapid $1\alpha,25\text{-(OH)}_2\text{D}_3$ -induced MAP-kinase activation may represent a form of cross-talk which could modulate the genomic pathways of $1\alpha,25\text{-(OH)}_2\text{D}_3$ (Song et al., 1998).

Moreover, the participation of nongenomic actions of $1\alpha,25\text{-(OH)}_2\text{D}_3$ in the cAMP/PKA signaling pathway was analyzed. Several studies indicated that the rapid stimulation of Ca^{2+} influx by $1\alpha,25\text{-(OH)}_2\text{D}_3$ in various cells was abolished by specific inhibitors of adenylate cyclase and PKA (de Boland and Norman, 1990a; Marinissen et al., 1994; Vazquez et al., 1995). In vitamin D-deficient chick soleus muscles, $1\alpha,25\text{-(OH)}_2\text{D}_3$ (10 pM–10 nM) elevated tissue cAMP levels within 45 s to 5 min and increased adenylate cyclase activity (Fernandez et al., 1990). Similarly, intracellular cAMP levels were raised in chick cardiac muscle and rat enterocytes (Massheimer et al., 1994; Selles et al., 1994).

Aside from the effects described above, $1\alpha,25\text{-(OH)}_2\text{D}_3$ has also been shown to elicit various other rapid nongenomic responses. Electrophysiologic studies were done in ROS 17/2.8 cells, where $1\alpha,25\text{-(OH)}_2\text{D}_3$ but not $1\beta,25\text{-(OH)}_2\text{D}_3$ promoted the rapid enhancement of Cl^- cur-

rent in a concentration-dependent manner (Zanello and Norman, 1997b). The hormone has also been reported to be involved in the stimulation of alkaline phosphatase activity (Ben Nasr et al., 1988) and phosphate transport (Karsenty et al., 1985; Suzuki et al., 1988).

Moreover, $1\alpha,25\text{-(OH)}_2\text{D}_3$ has been found to induce a reorganization of VDR from the cytosol to the nucleus occurring within 15 s to 30 min. This response was accompanied with an accumulation of cGMP (Barsony and Marx, 1991). Edelman et al. (1986) described a rapid change (within seconds) in the membrane potential of the renal proximal tubule from the amphibian *Necturus* resulting from the modification of Ca^{2+} -dependent K^+ channels by $1\alpha,25\text{-(OH)}_2\text{D}_3$. Furthermore, an inhibition of renal 25-dihydroxyvitamin D_3 1-hydroxylase induced by $1\alpha,25\text{-(OH)}_2\text{D}_3$ was found (Dick et al., 1990).

2. Vitamin D_3 Receptors for Rapid Signaling. The existence of a membrane receptor for $1\alpha,25\text{-(OH)}_2\text{D}_3$ has been suggested for several cells and tissues, such as chick intestinal cells (Nemere et al., 1994), NB4 human leukemia cells (Bhatia et al., 1995), ROS 24/1 cells (Cafrey and Farach-Carson, 1989; Baran, 1994), chondrocytes (Nemere and Farach-Carson, 1998), and tibial fracture-healing callus (Kato et al., 1998). A $1\alpha,25\text{-(OH)}_2\text{D}_3$ receptor located in the basal-lateral membrane of vitamin D-replete chick intestinal epithelium which was functionally correlated with transcaltachia has already been described in this review (see *Section III.B.2.*). Recently, an antibody directed against this membrane receptor (Ab99) was used for immunochemical studies in chick kidney and brain. These analyses have indicated that the membrane receptor for $1\alpha,25\text{-(OH)}_2\text{D}_3$ is present predominately in the lighter membrane fractions, including Golgi membranes and the plasmalemma (Jia and Nemere, 1999).

The $1\alpha,25\text{-(OH)}_2\text{D}_3$ analog [^{14}C] $1\alpha,25\text{-(OH)}_2\text{D}_3$ bromoacetate has been found to bind to a plasma membrane protein in ROS 24/1 cells that was identified to be annexin II (Baran et al., 2000). In addition, antibodies to annexin II diminished the binding of $1\alpha,25\text{-(OH)}_2\text{D}_3$ to partially purified plasma membranes and blocked the vitamin D-induced increases in $[\text{Ca}^{2+}]_i$ in these cells. Although supporting a challenging hypothesis, at present, these findings of tremendous potential relevance still await confirmation, even more than 2 years after publication of a related but unavailable abstract.

In summary, $1\alpha,25\text{-(OH)}_2\text{D}_3$ -induced effects on Ca^{2+} transport, $[\text{Ca}^{2+}]_i$, and phospholipase-associated signaling pathways, independent of genomic events, are mainly characterized in intestine and osteoblastic cells. With regard to receptors transmitting the nongenomic effects of this secosteroid, studies with analogs suggest that the ligand binding domains of the membrane vitamin D_3 receptor and the VDR are different. In search of a membrane vitamin D_3 receptor, the affinity ligand [^{14}C] $1\alpha,25\text{-(OH)}_2\text{D}_3$ bromoacetate was found to label two

distinct proteins in different cell types, with one of them being identified as annexin II.

F. Triiodothyronine

1. Rapid Effects of Triiodothyronine. In addition to the typical steroid hormones, triiodothyronine (T_3) has also been found to modulate gene activity through intracellular receptors closely related to the superfamily of intracellular steroid receptors (Evans, 1988). Moreover, thyroid hormones are also capable of generating biological responses by nongenomic mechanisms, and a great variety of rapid T_3 effects has been described.

As early as 1977, T_3 (10 nM) had been shown to produce a rapid increase in 2-deoxyglucose uptake in cultured chick embryo heart cells (Segal et al., 1977). More recent studies have confirmed this effect in heart and in various other organs of the rat (Segal, 1989b,c). In heart slices, the cycloheximide-insensitive stimulatory effect on 2-deoxyglucose uptake was evident within 11 min after addition of T_3 , with a maximal increase (about 80% above control) seen at a concentration of 1 nM. Associated with the 2-deoxyglucose uptake, a transient effect on Ca^{2+} uptake independent of extracellular Ca^{2+} has been described, which reaches a maximum after 30 s (Segal, 1990). Further effects of T_3 on cytosolic free Ca^{2+} levels have been studied in rat myocytes microinjected with aequorin. In this system, application of T_3 (1–100 nM) increased $[Ca^{2+}]_i$ within minutes (Lomax et al., 1991). Similar effects of T_3 involving Ca^{2+} membrane transport or $[Ca^{2+}]_i$ have been shown in thymocytes (Segal and Ingbar, 1984), rat liver cells (Hummerich and Soboll, 1989), and red blood cells (Lomax et al., 1991). In the latter, as well as in several other cell systems, it has been shown that T_3 stimulates plasma membrane and sarcoplasmic reticulum Ca^{2+} -ATPase activity, an effect involving increased Ca^{2+} efflux. This stimulation is likely to be mediated by PKC, which has also been described to be stimulated by T_3 (Davis and Davis, 1996). Recently, a rapid stimulation of the Na^+/H^+ antiporter by physiological concentrations of thyroid hormones (0.1 nM), resulting in a dose-dependent increase in intracellular pH, has been reported in L6 myocytes (Incerpi et al., 1999). A nongenomic effect of T_3 on the electrophysiological parameters of rat ventricular myocytes was seen using whole cell patch clamp techniques. Treatment of hypothyroid ventricular myocytes with T_3 (0.1 μ M) for 5 min significantly shortened action-potential duration (Sun et al., 2000). Thyroid hormones have also been described to rapidly stimulate O_2 consumption (Horst et al., 1989; Davis and Davis, 1996).

The clinical relevance of nongenomic T_3 action has recently been tested in a human study. In patients with severe heart failure and reduced free plasma T_3 , intravenous bolus application of T_3 did not change heart rate or blood pressure but significantly increased cardiac output and decreased systemic vascular resistance. The time course was too short to be compatible with genomic

mechanisms (Hamilton et al., 1998). T_3 -induced decrease of systemic vascular resistance may be explained by direct modulation of endothelium-independent vasoregulation (Park et al., 1997). In isolated cardiac myocytes, it has been demonstrated that T_3 can affect Na^+ currents—an effect which is compatible with the inotropic changes observed (Dudley and Baumgarten, 1993; Huang et al., 1999). This includes changes in Na^+ - Ca^{2+} exchange to produce increased contractility (Walker et al., 1995). The prompt response of certain voltage-gated K^+ channels to thyroid hormone may explain the findings that T_3 treatment lowered the prevalence of atrial fibrillation in postcoronary artery bypass graft patients (Klemperer et al., 1996; Sakaguchi et al., 1996). The relevance of nongenomic T_3 effects in cardiovascular physiology is further underlined by cycloheximide-insensitive, early modulation of β -adrenoceptor density by T_3 in cultured embryonic cardiac myocytes (Vassy et al., 1997), whereas the late up-regulation of β -adrenoceptors was sensitive to the inhibitor. This direct interaction may explain the observed T_3 -dependent sensitization of β -adrenergic inotropy (Walker et al., 1994).

2. Triiodothyronine Receptors for Rapid Signaling. High-affinity T_3 membrane-binding sites have been identified in membranes from rat liver (Pliam and Goldfine, 1977), thymocytes (Segal, 1989a), human placenta (Alderson et al., 1985), and VSMC (Ojamaa et al., 1996) exhibiting pharmacological properties consistent with an involvement in rapid, nongenomic action of T_3 .

As found for aldosterone- and progesterone-binding sites in microsomes from pig liver (see above), two classes of binding sites for T_3 , one with a high affinity ($K_d = 2$ nM) and a low capacity (~ 320 fmol/mg of protein), the other with a low affinity ($K_d = 18.5$ μ M) and a high capacity (~ 2.2 pmol/mg of protein), were detected in human placenta (Alderson et al., 1985). Similarly, in VSMC high- and low-affinity binding sites were found with K_d values of 0.01 and 61 nM, respectively (Ojamaa et al., 1996). The binding sites were found to be specific for T_3 in that other thyroid hormone analogs (D- T_3 , r T_3 , D- T_4 , and L- T_4) were less effective or ineffective in competing with bound [125 I] T_3 . In placenta, the affinity-labeling ligand *N*-bromoacetyl-[125 I] T_3 was found to specifically label a protein with an apparent molecular mass of 65 kDa as determined by SDS-PAGE. After gel filtration, the native labeled protein was found at a molecular mass between 140 and 150 kDa. In contrast, by the use of the same affinity label, a protein with a molecular mass of 27 kDa was detected in neuroblastoma plasma membranes (Goncalves et al., 1990).

In summary, T_3 elicits rapid effects on ion fluxes and electrophysiological events predominantly shown in the cardiac system, which may have a relevance in cardiovascular physiology. Several putative T_3 membrane receptors have been described in various tissues, but research in this regard is still in the beginning stages.

V. Two-Step Model for Steroid Action

In recent years, there has been growing evidence that rapid, nongenomic steroid actions occur in various *in vitro* and *in vivo* models. These nongenomic steroid actions are mainly characterized by a short delay of action (Gilligan et al., 1994; Christ et al., 1999b; Collins and Webb, 1999; Falkenstein et al., 2000) and the lack of inhibition of these effects by inhibitors of transcription and protein synthesis. Due to these differences of genomic and nongenomic actions of steroids, the classic genomic model of steroid action is not sufficient to explain the experimental and clinical results reviewed above. Potentially divergent pathways of action have been discussed from the beginning of steroid research; Clara Szego merits the honor of being the first to point out the diversity of steroid actions as early as the emergence of the genomic steroid action model (Pietras and Szego, 1975, 1977, 1999; Wehling et al., 1992b).

As steroids rapidly modulate intracellular signaling and second messengers such as cAMP have been demonstrated to influence steroid-induced transcription (Nordeen et al., 1994; Lim-Tio et al., 1997), a novel, two-step model steroid action has been proposed (Wehling, 1997; Christ and Wehling, 1998). This model comprises both the sluggish, genomic and the rapid, nongenomic modes of aldosterone action and, furthermore, proposes the modulation of the steroid-receptor-complex-initiated nuclear transcription and protein synthesis

by the nongenomic cellular signaling cascades and ion-transporter activities (Fig. 7).

The two-step model proposed here has been primarily developed for rapid aldosterone signaling, but similar models may be developed for other steroids, e.g., for estrogens or vitamin D₃ principally in a nearly identical form. The model not only adds a new, unrelated part to the traditional theory of steroid action but also addresses possible interactions between its components without challenging the unquestioned concept of genomic steroid action. Rapid steroid actions precede the traditional genomic mechanisms of steroid action. As second messengers [such as the transcriptionally active cAMP, pH, PKC, or PKA (Nordeen et al., 1994; Smith et al., 1996; Christ et al., 1999a)] or steroid-induced increases of kinase activity [MAP kinase (Endoh et al., 1997)] are known to influence genomic steroid action (Bunone et al., 1996), a possible interaction of rapid steroid signaling with genomic actions of the same steroids has to be assumed. Thus, the steroid and thyroid hormone may act through both mechanisms, mediating a wide range of cellular effects. This suggestion of intertwined pathways for nongenomic and genomic steroid action is further supported by findings on second messenger-induced modulation of glucocorticoid-related transactivation (Moyer et al., 1998). Furthermore, the agonist activity of the PR antagonist RU-486 is unmasked by independent stimulation of PKA (Nordeen et

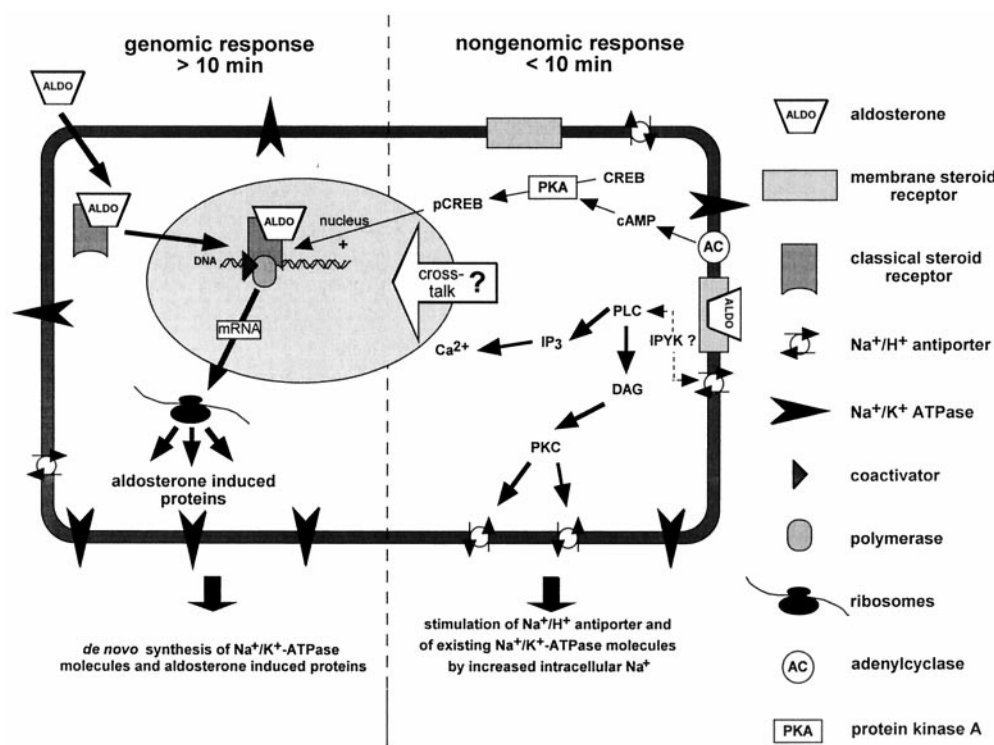


FIG. 7. Schematic presentation of the two-step model for steroid action comprising genomic and nongenomic actions of steroids exemplified for aldosterone. Steroids act via the classical genomic pathway by binding to intracellular steroid receptors, modulation of protein expression and, thus, cellular function (left side). The rapid, nongenomic pathway of steroid action involves membrane receptors, intracellular second messengers, and effector systems at the level of the plasma membrane. Changes of the intracellular signaling in turn may modify genomic modulation of steroid action. IPYK, intermediary protein-tyrosine kinase. Modified with permission from Christ and Wehling (1998).

al., 1993), pointing to a modulation of agonist and antagonist activity of certain steroids by changes of intracellular second messenger levels.

To take nongenomic steroid actions into account is one of many accomplishments in steroid research. Although beyond the scope of this review, which mainly focuses on nongenomic steroid action, a possibly even more intriguing progress has been made with regard to the understanding of nuclear steroid receptor biology. As pointed out in *Sections II.A. and III.A.*, the picture of classic steroid receptors merely representing transcription factors has been supplemented in many important aspects, which are at least as much intertwined as are genomic and nongenomic ones. These novel aspects of classic steroid receptors include the understanding of differential conformational switches induced by partial agonist/antagonists as well as the function of associated proteins, which on dissociation may transmit even nongenomic actions of the formerly "genomic" receptors. On the other hand, there are orphan receptors in the superfamily still searching for ligands, as well as known steroid receptors conveying effects even in the absence of ligands.

The two-step model may be expanded by the assumptions underlying the major aspects of the Mannheim classification of nongenomically initiated steroid effects. By introducing all known pathways by which steroids may induce nongenomic effects instead of restricting it to the original assumption of novel membrane receptors, the model would still comprise nongenomic and genomic events. The cellular consequences of nongenomic steroid actions, including the cross-talk with genomic effects, are comparably insensitive to the particular pathway involved, with the resulting cellular pleiotropism of steroid action being the major achievement in the development of current scientific understanding.

In summary, the two-step model of steroid action comprises both rapid, nongenomic and the delayed, genomic actions of steroids and their interdependence. The specificity of steroid action may be caused at least in part by steroid-induced, rapid modulation of second messengers and their influence on nuclear transcription.

VI. Clinical Perspectives

Steroid hormones are involved in almost all physiological processes in the human body. To name a few, conception, intrauterine fetal development, bone maturation, immune system regulation, water and electrolyte homeostasis, and CNS activity are among the most striking examples for development and maintenance of human life in which steroids play a crucial role. The characterization of the mechanisms that mediate steroid actions has recently focused on nongenomic effects and an intracellular cross-talk that engages both nongenomic and genomic mechanisms. The broad array of mechanisms by which steroids mediate their biological effects provides multiple

opportunities for pharmacological interventions in various clinical conditions. In addition to well established genomic steroid effects, nongenomic modulation of physiological and pathophysiological processes opens perspectives for pharmacological intervention far beyond today's application.

A. Cardiovascular Pharmacology

Aldosterone has only recently re-entered the center stage of research interest due to the RALES findings, a study in which a beneficial effect of spironolactone in heart failure could be demonstrated (Pitt et al., 1999). The detrimental effects that aldosterone apparently conveys in heart failure may partly be mediated via nongenomic mechanisms. A nongenomically mediated increase in systemic vascular resistance as well as a decrease in cardiac output and baroreceptor sensitivity (Christ et al., 1999b) may constitute unfavorable aldosterone effects in hypertension and heart failure. Specific antagonists of a putative membrane receptor for aldosterone would provide additional options in the pharmacological treatment of these diseases.

Coronary artery disease, one of the main causes of death in developed countries, can also be considered as a target for pharmaceutical intervention with steroids because of nongenomically mediated mechanisms. Beneficial therapeutic effects could be derived from the potential of 17β -estradiol and progesterone to immediately relax small and large arteries (Kausar and Rubanyi, 1997; Kakucs et al., 1998). Estrogen, but not gestagen, hormone replacement may confer additional therapeutic cardiovascular benefits due to a favorable modulation of heart rate variability, an indicator of sympathetic balance and as such a predictor for cardiovascular mortality (Christ et al., 1999c). Furthermore, epidemiological studies have repeatedly suggested that estrogens protect against the development of coronary artery disease in postmenopausal women, but the mechanisms involved largely remain to be elucidated. An interesting recent study suggests that nongenomically mediated Ca^{2+} -antagonistic effects of plant-derived estrogens may relax coronary arteries of rabbits *in vitro* (Figtree et al., 2000). This may be considered a mechanistic link, possibly explaining some of the proposed beneficial effects of estrogens in the prevention of coronary events. The development of potent modulators of nongenomically mediated effects on vasoreactivity and sympathetic balance could therefore represent an immeasurable gain, especially if such agents are devoid of genomically mediated side effects of female sex steroids.

B. Reproductive Pharmacology

The exciting findings that progesterone ameliorates the human sperm acrosome reaction via nongenomic mechanisms (Baldi et al., 1999; Forti et al., 1999) have propelled speculations of improving support to select the appropriate assisted reproductive technique in subfer-

tile patients. Recently, the binding of progesterone to sites on the acrosomal membrane has been proposed as a valuable predictor for the outcome of in vitro fertilization. With increasing binding of progesterone to sperm, there was a higher success rate of fertilization (Fukui et al., 2000).

It may be speculated that an impaired sperm acrosome reaction caused by reduced sensitivity to natural progesterone may be enhanced by progesterone at high concentrations or by agents still to be discovered that improve receptor binding and/or sensitivity to progesterone, possibly by allosteric modulation. An additional target for intervention could be the recently discovered functional estrogen receptor in the surface of human sperm (Baldi et al., 2000). Estrogens may directly inhibit the progesterone-stimulated acrosome reaction. It remains to be elucidated if the proposed direct cross-talk between membrane receptors for estrogen and progesterone is pathophysiologically important for an appropriate timing of capacitation and the acrosome reaction. If so, inhibiting the estrogen receptor while enhancing the effects of progesterone may present a hypothetical pharmacological concept for successfully intervening in male infertility.

C. Endocrinological Pharmacology

Non-insulin-dependent diabetes mellitus is a clinical condition in which pharmacological intervention to regulate blood glucose levels is restricted to biguanides, glitazones, and sulfonylureas. An additional strategy may evolve from the insulinotropic effect of a vitamin D analog through nongenomic signal transduction (Kajikawa et al., 1999). Even though this result needs to be evaluated in respect to its clinical relevance, it is tempting to speculate about a specific interaction of vitamin D₃ or its analogs with a still putative membrane vitamin D₃ receptor of pancreatic β -cells that may enhance insulin secretion without entailing the side effects known for biguanides, glitazones, or sulfonylureas. Whether annexin II is indeed a functional membrane receptor for nongenomic actions of vitamin D₃ (Baran et al., 2000) is certainly one of the key questions to be answered soon. If so, it would be important to know whether it can be found in the pancreas, and whether it is able to mediate rapid actions of vitamin D₃ there.

Type I diabetes mellitus may also be a target for future vitamin D research. By as yet unknown mechanisms, vitamin D supplementation during infancy seems to protect against the development of insulin-dependent diabetes mellitus. It has been speculated that an immune modulation yet to be characterized might contribute to protecting against or arresting an ongoing immune process that otherwise eventually leads to type I diabetes mellitus (anonymous, 1999).

Nongenomic effects on the immune system may also be testosterone-dependent. Classic ARs in splenic T cells are genomically unresponsive to testosterone. In con-

trast, the cell surface receptors for testosterone are functionally active, enabling T cells a nongenomic response to testosterone (Benten et al., 1999a). This finding suggests an as yet unexploited option for a modulation of T cell activity with possible applications in proliferative and infectious diseases. As an example, the binding of testosterone to surface receptors of splenic T cells which triggers an immediate Ca²⁺ influx may be the initial event in the testosterone-induced susceptibility to *Plasmodium chabaudi* malaria in mice (Benten et al., 1997). If this finding could be substantiated in humans for other *Plasmodium* species, it may be speculated that selective inhibition of testosterone T cell surface receptors may present a new way of preventing malaria infections.

D. Neuro-/Psychopharmacology

Neuroactive steroids alter neuronal function through nongenomic and genomic mechanisms. An intracellular cross-talk seems to be particularly important in mediating complex CNS functions, but its experimental accessibility has proven to be extremely difficult. Additional work will have to be carried out to clearly characterize structure-activity relationships of various neuroactive steroids at different neurotransmitter receptors. Possibly, specific agonists and antagonists could be developed to be used in future functional studies and consequently in therapeutic applications. Even in light of today's knowledge, exciting therapeutic possibilities emerge for an array of neurologic and psychiatric disorders. However, research will have to focus on the characterization of the effects of neuroactive steroids in humans in vivo because most of today's knowledge in this area derives from in vitro or animal studies.

Despite this gap of knowledge, applications for the therapeutic use of neuroactive steroids have been proposed in anesthesia, epilepsy, anxiety, depression, insomnia, migraine, memory impairment, and drug dependence. Apart from mechanistic studies investigating an allosteric modulation of neurotransmitter receptors and genomic regulation of these receptors by steroids, epidemiological evidence suggests a disequilibrium of neuroactive steroids in neurological and psychiatric disorders. In some instances such a disequilibrium may be crucial for the natural history and therapy of a disease.

Specific applications for neuroactive steroids for epilepsy have arisen on the horizon. In preclinical studies, the efficacy of allopregnanolone, pregnanolone, and the new synthetic GABA_A receptor agonist ganaxolone has been promising. Especially in the prevention of experimentally induced seizures in rats, neuroactive steroids offered advantages over the conventional anticonvulsant drugs diazepam and valproate, which have so far been unsatisfactory (Gasior et al., 2000; Vanover et al., 2000). First applications of neuroactive steroids as sedatives and anesthetics have proven effective but are still fraught with adverse drug reactions. In anxiety disorder

ders, clinical applications for neuroactive steroids could be reached soon. On the one hand, an allosteric modulation of the GABA_A receptor in itself may confer anxiolytic properties in humans; on the other hand, an adjunctive treatment of benzodiazepines and neuroactive steroid acting as coagonists at the GABA_A receptor may prove beneficial. This may particularly hold true because neuroactive steroids may alleviate the symptoms of benzodiazepine withdrawal.

The 5-HT₃ receptor may also be tied to future clinical applications of neuroactive steroids. The 5-HT₃ receptor is known to be involved in the development of pregnancy-associated nausea (Broussard and Richter, 1998). If research continues to illuminate the connection between levels of sex steroids that are altered during pregnancy and may contribute to nausea, a second step may lead to safe and effective therapies for hyperemesis gravidarum which are not yet readily available (Jewell and Young, 2000).

The proposed neuroprotective and memory-enhancing effects of neuroactive steroids probably involve a variety of neurotransmitter receptors and metabolites. Today we only poorly comprehend the complexity of mechanisms involved in the effects of neuroactive steroids. Evidence so far is sparse that the neuroprotective, anxiolytic, anticonvulsant, and memory-enhancing properties, among others, of neuroactive steroids observed in rodents are also applicable to humans. A deeper understanding of intracellular cross-talk initiated by neuroactive steroids via nongenomically and genomically mediated neuron modulation is therefore desperately needed. In addition, future work has to focus on the complex interaction of agonistic and antagonistic properties of neuroactive steroids at different receptors. Only this may lead to the development of pharmacological agents with clear-cut pharmacological profiles. Research that broadens our knowledge of fast steroid action and its clinical implication should be strongly encouraged.

VII. Conclusions and Outlook

Rapid responses of steroid hormones and related compounds have been described—although to varying depths—at all biological levels spanning from intracellular signaling to whole animal and even to human physiology. Although the general signaling pathways utilized are often similar, each steroid seems to display its own facets, and the mechanisms of rapid steroid signaling are not uniform. Moreover, some responses are described to be restricted to particular tissues or organs. Therefore, specific nongenomic actions seem to depend on the type of steroid, cells, tissues, or species used.

Because second messengers are known to influence genomic steroid action, a possible cross-talk of rapid steroid signaling with genomic steroid actions has also been hypothesized. Steroid hormones may act through both mechanisms simultaneously, and these mecha-

nisms appear to be important comediators of the wide array of cellular steroid effects.

It is obvious that many aspects of nongenomic steroid action still require continuous intensive research, and essential clues for their understanding are still lacking. This also includes the still unaccomplished cloning of the putative membrane receptor initiating rapid steroid signaling. There are preliminary findings on membrane proteins that bind steroids (mPR, annexin II), but none has been definitively proven to represent a steroid membrane receptor responsible for rapid action. Clearly, the cloning of such a receptor would be a major advance, but it should not be forgotten that the definition of the physiological, pathophysiological, and, last but not least, clinical relevance of novel aspects of steroid action should be another target of paramount importance. This may even be achieved without the receptors cloned, although the latter would definitely also ease clinically oriented research.

Novel aspects of “classic” steroid receptors such as nontranscriptional activities or constitutional, nonligand-mediated effects will certainly add more detailed knowledge to the complex plot of steroid physiology, and integrative views must consider all major aspects to fully utilize the emerging potentials of steroids in physiology, pathophysiology, and therapeutics.

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