Nongenomic Steroid Action: Controversies, Questions, and Answers

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Loesel, Ralf M., Elisabeth Falkenstein, Martin Feuring, Armin Schultz, Hanns-Christian Tillmann, Karin Rossol-Haseroth, and Martin Wehling. Nongenomic Steroid Action: Controversies, Questions, and Answers. Physiol Rev 83: 965–1016, 2003; 10.1152/physrev.00003.2003.—Steroids may exert their action in living cells by several ways: 1) the well-known genomic pathway, involving hormone binding to cytosolic (classic) receptors and subsequent modulation of gene expression followed by protein synthesis. 2) Alternatively, pathways are operating that do not act on the genome, therefore indicating nongenomic action. Although it is comparatively easy to confirm the nongenomic nature of a particular phenomenon observed, e.g., by using inhibitors of transcription or translation, considerable controversy exists about the identity of receptors that mediate these responses. Many different approaches have been employed to answer this question, including pharmacology, knock-out animals, and numerous biochemical studies. Evidence is presented for and against both the participation of classic receptors, or proteins closely related to them, as well as for the involvement of yet poorly understood, novel membrane steroid receptors. In addition, clinical implications for a wide array of nongenomic steroid actions are outlined.

I. INTRODUCTION

A. Early Work

In 1942, Hans Selye published a study on the correlation between chemical structure of steroids and their pharmacological action (437). In this study, rats were administered a number of different steroids intraperitoneally, and very rapid anesthetic effects were noticed. In addition, the main hormone effects known at this time, namely corticoid, folliculoid, luteoid, and testoid action, were investigated. Whereas anesthesia occurred within minutes, the other hormone effects required several hours or days to become visible. In addition to the drastically...
differing time scale, the other striking finding in this work was the variation of the relative magnitude between the rapid anesthetic and the delayed “main” hormone action among the compounds tested. In these days, very little was known about the mechanisms involved in steroid hormone action, but it has been concluded that “any one of these activities may be exhibited . . . irrespective of any other hormonal properties” which in contemporary perception would be interpreted as the involvement of different receptors and pathways of action. The main hormone action is now known to be mediated by “classic” steroid receptors, mainly located in the cytosol and the nucleus, acting on the genome.

Two decades later, in 1963, Klein and Henk (233) demonstrated acute cardiovascular effects of aldosterone in men. Within 5 min after administration of the steroid, peripheral vascular resistance and blood pressure increased whereas cardiac output decreased. The short time frame suggests a nongenomic mechanism, as explained below. In vitro effects of aldosterone at physiological concentration on Na⁺ exchange in dog erythrocytes were reported almost simultaneously (462). This experiment very clearly demonstrates the existence of nongenomic pathways for steroid action, as mammalian erythrocytes lack a nucleus. Other rapid, presumably nongenomic, steroid effects have been demonstrated later (125), and when the genomic steroid model was just emerging, Pietras and Szego (375, 376) already underlined (125), and when the genomic steroid model was just emerging, Pietras and Szego (375, 376) already underlined

the diversity of steroid action.

B. Genomic Versus Nongenomic Action

According to the generally accepted theory of steroid action, steroid molecules enter the cell, either passively by diffusion through the membrane or assisted by any transporter; subsequently, intracellular receptors, located in the cytosol or in the nucleus, bind the steroid, thereby undergoing a conformational change. This leads to the dissociation of accessory proteins, which have previously maintained the receptor in an active form, and to an increase in DNA affinity of the DNA binding domain. The steroid-receptor complex migrates to the nucleus, where it influences the transcription of genes into mRNA. Finally, mRNA is translated into protein molecules, which ultimately exert biological functions. There are several reviews describing these events comprehensively (36, 37). This pathway is called “genomic,” because it involves action on the genome, and from the sequence of events several properties become obvious.

1) The time required to fully activate this pathway is comparatively long. For aldosterone, early genes are differentially expressed 1 h after steroid addition (501). Furthermore, protein synthesis requires additional time, and to exert a given biological function, significant amounts of the respective protein must be generated. Clinically, i.e., at the level of the whole organism, effects are usually seen after hours or even days.

2) This pathway is sensitive to inhibitors of transcription (such as actinomycin D) as well as inhibitors of translation (cycloheximide).

In contrast to the genomic pathway outlined above, nongenomically mediated phenomena often occur with very short lag time. As examples, progesterone induces a significant increase in intracellular calcium in spermatozoa within seconds (58), and aldosterone activates the Na⁺/H⁺ antiporter within 1 min in colonic crypts (526).

Even more striking are the examples of steroid effects that occur in cells without a functional nucleus, such as erythrocytes, platelets, or spermatozoa. All these cells rapidly respond to stimulation by various steroids; details will be described in the following sections.

As the number of reports on nongenomic steroid effects has grown tremendously in the past two decades, it has become likely that the action of steroids in living cells is mediated by various pathways rather than a single uniform mechanism. The Mannheim classification scheme (142) is helpful in describing and separating potential mechanisms and understanding the distinction between classic and nongenomic steroid action.

The scheme (Fig. 1) divides pathways of steroid action by several criteria. The main groups A and B differ for the requirement of a partner agonist to elicit a steroid response. Within these groups, steroid action can be further classified as nonspecific (I) or displaying ligand specificity (II). According to the identity of the receptors mediating the specificity in the latter category, a distinction is made between the classic steroid nuclear receptor (a) and other, nonclassic steroid receptors (b). On the basis of current findings, the classic steroid receptor itself is able to drive signaling cascades.

Examples for every class contained in the scheme are not yet known, and within the classes the specific phenomena vary between species, tissue, cell type, and, obviously, the steroid involved.

II. RECEPTORS

A. The Steroid Nuclear Receptor Superfamily: Classic Receptors

Steroid molecules all share the minimum skeleton of four fused rings, which is not only true for the steroids known from mammals, but also for the structurally related hormonally active substances from insects and plants (e.g., ecdysone and the brassinosteroids). This structural relation coincides with a structural similarity in their animal receptor proteins, which form a protein superfamily together with the receptors for thyroid hor-
mones, retinoic acid, and vitamin D (37, 134). Generally, a classic steroid receptor comprises a NH₂-terminal domain of considerably variable length with possible modulator function, a highly conserved DNA binding domain, and a ligand binding domain of ~220–250 amino acids length at the COOH terminus (35). Isoforms originating from differing promoter usage and other variants have been described for many steroid receptors, but their functional role is not always known. More than 65 genes coding for nuclear receptors have been identified in animals (104), although their ligands are not known in all cases (orphan receptors). Some of these orphan receptors are likely to bind steroids as well, such as the farnesoid X receptor (266), which binds bile acids, a fact that may indicate an even broader scope of steroid action than previously known. Other orphan nuclear receptors comprise the peroxisome proliferator-activated receptors (PPAR), which are involved in regulating glucose and lipid homeostasis. In their unliganded state, nuclear receptor molecules are associated with chaperone proteins that are thought to keep them functional.

B. Which Receptors Mediate Nongenomic Action: The Controversy

The commonly accepted mechanism for steroid hormone action, as found in all related textbooks, comprises the following steps: steroid molecules enter the cell and bind to their (classic) receptors described in the previous section. The receptor-steroid complex then translocates to the nucleus, if not already there, and modifies gene transcription. As detailed in the previous sections, there are numerous physiological effects that cannot be mediated by action on the genome, i.e., transcription and translation, as judged from their insensitivity toward appropriate inhibitors and/or the short time frame in which the responses occur.

Such nongenomic effects obviously involve receptors as well, if not simply indicating nonspecific steroid effects, e.g., on the membrane. A controversy has started, whether this task is fulfilled by classic receptors as well, thus representing additionally, hitherto unknown functionality, or by different receptors unrelated to them. The claim of distinction has often been made on the basis of pharmacological properties, as a specific ligand binding domain is believed to have a characteristic ligand selectivity pattern, and should, therefore, be at least very similar for genomic and nongenomic action if both were mediated by the same receptor. Unfortunately, physiological responses to steroids involve living cells capable of and using additional mechanisms that may interfere with the determination of a pharmacological profile and its correlation to ligand selectivity of the isolated receptor. A very instructive example is the hydroxysteroid dehydrogenase reaction in conjunction with the mineralocorticoid receptor. Thus some distinctions and classifications merely based on pharmacology deserve reevaluation. It must be noted that, unlike the classic steroid receptors, only few other, novel receptors linked to a defined steroid effect have been rigorously identified yet, such as receptors for neurotransmitters that are modulated by steroids (see sect. dD5), the maxi-K channels (493), the Na⁺⁻K⁺-ATPase (which binds cardiotonic steroids), and the brassinosteroid receptors in plants.

Both the discovery of additional phenomena interfering with cellular steroid action and the yet moderately successful identification of alternative receptors have brought new life to the controversy of how nongenomic steroid action is mediated. In the following sections, evidence for either part is presented and critically discussed.

C. Evidence for the Existence of Receptors Distinct From Classic Receptors: Some Examples

Some evidence has been compiled from experimental systems that indicate the participation of receptor molecules that are not identical or closely related to the
cytosolic, classic steroid receptors. This does not necessarily mean that rapid, nongenomic phenomena are always mediated by nonclassic receptors, e.g., rapid estradiol action is likely to be transduced by its classic receptor (estrogen receptor, ER; see sect. II/D/3) in many cases, and the classic progesterone receptor (PR) was shown recently to drive rapid signaling cascades. However, there are numerous physiological steroid responses that are incompatible with exclusive classic receptor signaling for reasons which shall be detailed here.

1. Knock-out mice

The most straightforward way to investigate the involvement of a protein in a physiological process is the generation of an organism that has the respective gene disrupted or knocked out. The mineralocorticoid receptor (MR) knock-out mice MR/−/−, obtained by gene targeting by Berger et al. (48), die between day 8 and 13 after birth, with a markedly reduced weight and a severe dehydration due to failure of sodium reabsorption. They show all signs of pseudohypoaldosteronism, such as hyperkalemia, hypotension, and a strongly activated renin-angiotensin-aldosterone system.

Measurements of intracellular calcium were performed in wild-type mice skin fibroblasts and in fibroblasts from MR-knock-out (KO) mice after stimulation with aldosterone (207). The basal values of intracellular Ca²⁺ concentration ([Ca²⁺]i) were not significantly different in wild-type and KO fibroblasts, respectively. After addition of 10 nM aldosterone, the [Ca²⁺]i increases in wild-type and mutant cells were both significant versus baseline levels, with a trend to even larger and prolonged signals in cells from KO mice. The response was almost complete within 1 min. In contrast, neither stimulation with 10 nM nor with 1 μM cortisol showed a significant increase of intracellular calcium in wild-type cells.

In addition to intracellular calcium measurements, cAMP levels were determined and basal levels found to be similar in wild-type and KO mice (207). Incubation of the cells with 10 nM aldosterone increased intracellular cAMP levels 2.2-fold within 1 min in wild-type fibroblasts and interestingly even more in KO mice cells (10.6-fold increase).

Preincubation of the wild-type mice cells with 10 μM spironolactone, an antagonist at the classic mineralocorticoid receptor, did not significantly decrease aldosterone-induced effects on intracellular cAMP levels (207). Because aldosterone was used at physiological concentration, unspecific membrane interaction is unlikely.

However, in vitamin D receptor KO cells, nongenomic effects of 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] such as opening of ion channels were not detectable (A. W. Norman, personal communication).

Further experiments involving classic receptor KO animals include ERα KO mice, which still exhibit estradiol-induced extracellular regulated kinase (ERK) phosphorylation (483). This observation, however, awaits confirmation in a double (ERα/ERβ) KO animal. Other experiments conducted with neurons from ERα KO mice demonstrated that the estradiol-induced potentiation of kainate-induced currents is still present (193). Preincubation with ICI 182780, which is supposed to block both ERα and ERβ, did not blunt the estrogen effect. The sensitivity toward the cAMP thio analog adenosine 3’,5’-cyclic monophosphothioate, Rₚ isomer (Rₚ-cAMPS), suggests a cAMP-dependent pathway.

In ovariectomized and estradiol-primed PR KO mice, intravenous infusion of progesterone increased lordosis within 10 min, a phenomenon identical to that seen in wild-type mice (160).

The rat analog of the porcine progesterone binding membrane protein (mPR), 25-Dx (436), has been shown to be expressed to a higher level in female PR KO mice than in their wild-type cognates (238). Together with other data on the regulation of its expression, a mechanism has been suggested by which 25-Dx expression is repressed through activated PR, which shares some similarities with regulation in the case of aldosterone.

2. Pharmacology

A) Vitamin D₃. Vitamin D₃ itself is biologically inert, and it needs to be metabolized to 1,25(OH)₂D₃ and other metabolites to achieve its biological activity.

It is well established that 1,25(OH)₂D₃ can stimulate biological responses via signal transduction pathways that utilize the classic nuclear receptor for 1,25(OH)₂D₃ (vitamin D receptor, VDR) to regulate gene transcription. It belongs to the superfamily of steroid receptors (see sect. II/A) and has been cloned, sequenced, and functionally expressed (290). In addition, there is considerable evidence that 1,25(OH)₂D₃ can generate rapid, nongenomic biological responses via different signaling pathways (for details, see sect. II/G).

The naturally occurring isomer of 1,25(OH)₂D₃ is the α-isomer [1α,25(OH)₂D₃]. Compared with steroid molecules in the narrow sense, which have a rigid skeleton, it is unusually flexible in its conformation due to the missing 9–10 carbon-carbon bond and easily undergoes a cis/ trans-isomerization at the 6–7 carbon-carbon bond in the seco B-ring.

In the course of examination of 1,25(OH)₂D₃ binding to VDR and a putative membrane receptor, through which it might induce genomic and nongenomic responses, respectively, a series of analogs locked in either the cis- or the trans-conformation have been generated. The cis-locked conformers activate the rapid, nongenomic pathways but bind poorly to the nuclear receptor and are only weak agonists for genomic responses (145, 340). In addi-
tion, the diastereomer 1β,25(OH)₂D₃, while unable to block the genomic effects of 1α,25(OH)₂D₃, was found to be a potent inhibitor of transcalcitriol (the rapid, non-genomic stimulation of calcium transport) (339) and other effects (343). Extensive studies on structural features of vitamin D analogs with regard to their differential activities in the genomic and nongenomic pathways have been elaborated in chick intestine and osteoblast/ROS 17/2.8 cells (20, 122, 341 and others). Aside other analogs, the 6-s-cis-isomer of 1,25(OH)₂D₃ has been found to specifically stimulate nongenomic effects such as transcalcitriol, but not genomic biological responses. EC₅₀ values for those effects are in the subnanomolar, thus physiological, range.

A series of vitamin D₃ analogs have been identified that activate only subsets of the biological responses of the hormone (342, 343) (see Fig. 2). 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 (338) and are only weak agonists for the genomic responses (63, 542). In contrast, trans-locked analogs did not promote transcalcitriol even at concentrations up to 6.5 nM, and a further analog, the 1α,25(OH)₂D₃-epimer, has been found to antagonize the 1α,25(OH)₂D₃-mediated response (339). The addition of hybrid analogs of 1α,25(OH)₂D₃ modified at the A-ring and the C,D-ring side chain were described to have an influence on proliferation rate, proteoglycan production, and protein kinase C (PKC) activity of rat chondrocytes (66). However, effective binding of these analogs to the classic VDR was only 0.1% relative to genuine 1α,25(OH)₂D₃.
The interconversion between the 6-s-trans- and 6-s-cis-forms has a low energy barrier and therefore occurs rapidly in solution at room temperature, generating multiple intermediate conformers. As yet, the true equilibrium ratio of the 6-s-trans- and 6-s-cis-conformers of any vitamin D seco steroid, including 1,25(OH)2D3, has not been rigorously determined. It has been estimated by computational methods that 88–99% of 1,25(OH)2D3 exists as the 6-s-trans-conformation, with only 1–12% existing in the 6-s-cis-form. Obviously, not all 1,25(OH)2D3 diastereomers are as active as 1α,25(OH)2D3. With regard to the potency for transcaltachia and for 45Ca2+ influx in ROS 17/2.8 cells, the diastereomers are different (342). Due to the facile interconversion of the 6-s-trans- and 6-s-cis-conformers of 1,25(OH)2D3, there exist kinetically competent amounts of all conformers available to interact with any receptors which may, in turn, be linked to the generation of biological responses.

B) ALDOSTERONE. The pharmacology of the classic MR has been studied in great detail. Agonists include the natural and most potent ligand aldosterone, but also deoxycorticosterone, corticosterone (physiologically important in rodents), and cortisol. While aldosterone is bound by the isolated MR with a dissociation constant (Kd) ~1 nM, cortisol binds to the same receptor with almost equal affinity (11, 38, 239, 324). However, under physiological conditions, the MR is thought to be protected by several mechanisms that metabolize “undesired” ligands to compensate for its low intrinsic specificity (346, 389).

The inhibition of MR activation is beneficial in a number of diseases, and therefore numerous drugs with MR antagonist activity have been synthesized and studied, with spironolactone being the most popular. It has been shown that under in vitro conditions, the presence of a lactone ring spiro to the D ring is a prerequisite for high-affinity binding (369), which is present in spironolactone as well as in its major metabolite canrenone. In vivo, the open-ring compound canrenate is rapidly equilibrated with its conversion product canrenone, an active MR antagonist, while canrenate is only a very weak MR ligand in vitro (369) (see Fig. 3).

The relative agonistic and antagonistic activities of the drug compounds described above have often been used to distinguish between mechanisms involving the classic MR and other pathways on a pharmacological basis. Whereas aldosterone induced phenomena that can be blunted by the addition of spironolactone (or other lactone antagonists) point to the involvement of the classic MR, spironolactone insensitivity has reasonably been interpreted as evidence for the presence of a different receptor.

Numerous studies have described aldosterone effects that could not be blocked by spironolactone. In addition, many of these effects were not elicited by cortisol despite its high affinity to the MR, which has often been interpreted as different specificity. This hypothesis has been challenged, however, by a recent study which demonstrated that the previously inactive cortisol becomes equally active as aldosterone in rapidly elevating intracellular pH after addition of carbenoxolone, a 11β-hydroxysteroid dehydrogenase inhibitor (5). This finding is a striking argument for the involvement of classic MR. The rise in pH could not be blunted, however, by spironolactone at very high excess, nor by the spiro lactone RU 26752. The open-ring congener of the latter, RU 28318, was able to block the aldosterone-induced effects (see Fig. 4). From binding studies it is known that RU 28318 is only a very weak ligand at the MR, as is canrenone which has almost two orders of magnitude lower affinity than its counterpart canrenone (369). Both are, however, almost equally effective in vivo, as they are converted into each other and an equilibrium is rapidly established, and the same is true for RU 28318. The phenomena described by Alzamora et al. (5) thus share some of the properties of the classic MR, which is sometimes taken as evidence for its participation (163), but the sensitivity toward inhibitors is entirely reversed. As ligand selectivity is governed by molecular attraction and repulsion, it is a function of receptor (or its ligand binding domain’s) structure. Thus it is hard to imagine that the same or a very similar ligand binding domain preserves only part of its selectivity, but reverses the other (inhibitor) part.

Evidence based on pharmacology generally is not regarded as compulsory, due to the many cellular events that are blurring the observation. However, to explain the data from the study mentioned above by involvement of classic MR, a cellular mechanism needs to be hypothesized that selectively transforms a (in vitro) weak antagonist (RU 28318) into a strong one (possibly the closed-ring RU 26752), but at the same time prevents the high-affinity antagonists spironolactone and RU 26752 (if added directly) from acting at the MR. Given the ligand (and antagonist) selectivity pattern seen here, a distinct nonclassic receptor with different selectivity is the more convincing explanation.

3. Systems where the classic receptor has not been demonstrated

Several systems have been thoroughly investigated to demonstrate one or the other classic receptors. The methods employed mainly comprise immunochemistry with antibodies raised against the respective receptor, and RT-PCR. Obviously, a negative proof in such cases is more difficult, as the failure to detect the protein in question or its mRNA could also be a matter of sensitivity, or very low levels that are (false) detected may be inferred by impurities in samples. A convincing example is the testosterone action on [Ca2+]i in IC-21 macrophages (46). In these cells, the classic androgen receptor (AR) was not
detectable by several experimental approaches: immuno-
staining, Western blotting, as well as RT-PCR using sev-
eral primer pairs. However, testosterone and testoster-
one-BSA-fluorescein isothiocyanate (FITC) conjugate
both induced a \([Ca^{2+}]/H_1\) response, and confocal techniques
demonstrated exclusive membrane localization of the flu-
orescent steroid conjugate (for a critical discussion of
steroid conjugates, see sect. V). The human prostatic cell
line PC3 also was reported to be devoid of AR (270). In
these cells, testosterone still induces rapid transient
\([Ca^{2+}]/H_1\) increases.

In neuronal cells lacking a functional ER, 17\(\beta\)-estra-
diol still activated mitogen-activated protein kinase
(MAPK) signaling and augmented the release of amyloid
\(\beta\)-precursor protein (279).

Some other examples are discussed in the sections
on individual steroids.

A different, yet striking example is the rapid effect of
aldosterone on calcium influx and intracellular cAMP in
human proximal tubular cells (unpublished results).
These cells apparently do not have a functional MR, and
these effects thus strongly suggest involvement of a non-
classic receptor.

D. Additional Evidence For and Against
Distinct Receptors

This section serves to summarize evidence for the
involvement of either classic or nonclassic receptor mol-

FIG. 3. Molecular structures of aldoste-
one, the physiological ligand of the mineralo-
corticoid receptor (MR), and some of its antag-
onists. The closely related cortisol binds to iso-
lated MR with roughly equal affinity but is
converted to an inactive form in vivo. The
closed-ring compounds spironolactone, can-
renone, and RU 26752 effectively displace aldo-
sterone from MR, thus explaining their antago-
nistic action, whereas the open-ring compounds
canrenoic acid and RU 28318 are known to be very
weak competitors. In vivo, however, an equilib-
rium forms between open-ring and closed-ring
structures (indicated by arrows). Interestingly,
the open-ring compound RU 28318 inhibits non-
genomic aldosterone action, whereas spirono-
lactone is inactive (cf. Fig. 4).
molecules, or even different mechanisms such as allosteric action.

Several enzymes have been shown to be susceptible to activity modulation by steroids, e.g., mitochondrial ATPase (541). However, the concentrations required often exceed the physiological range by far. Such mechanisms shall not be discussed in detail here.

1. Glucocorticoids

Specific nongenomic glucocorticoid effects have been suggested for a long time to be mediated via membrane-bound receptors, whereas nonspecific nongenomic effects are thought to occur due to physicochemical membrane interactions.

Membrane-binding sites for different glucocorticoids have been described in many tissues and cells. In plasma membranes from chicken liver, mouse liver, and rat liver, specific binding sites for cortisol could be demonstrated (218, 485, 489). In rat liver plasma membranes, two types of binding sites for cortisol have been detected, a high-affinity site with low binding capacity and a low-affinity site with high binding capacity. Further analysis of the high-affinity binding site by SDS-PAGE showed two protein subunits of 52 and 57 kDa, respectively, and binding affinities distinct from those of the glucocorticoid receptor (GR) (218). In addition, the binding of corticosterone to murine and rat liver plasma membranes, to rat kidney and rat brain plasma membranes, and to calf adrenal cortex plasma membranes has been shown (219, 486, 487 and others).

Another glucocorticoid responsive site has been shown in a highly purified rat liver plasma membrane fraction. This site mediates active transmembrane transport of corticosterone, and it does not seem to be a member of the ABC transporter or multidrug resistance transporter superfamily (4, 242).

In 1991, Orchinik et al. (352) identified a corticosteroid receptor in synaptic membranes prepared from taricha granulosa brain that showed high-affinity binding of $^3$H-labeled corticosterone (352). Furthermore, the affinities of corticoids for this binding site were linearly related to their potencies in rapidly suppressing male reproductive behavior, thus suggesting its participation in the regulation of behavior. Evidence for the rapid modulation of Taricha behavior by binding of steroid hormones to specific membrane-associated receptors is reviewed in detail by Moore and Orchinik (312) and Moore et al. (313).

In equilibrium saturation binding studies and in titration studies, nonhydrolyzable guanyl nucleotides were able to inhibit the binding of $^3$H-corticosterone to neuronal membranes. The addition of Mg$^{2+}$ enhanced both the equilibrium binding of $^3$H-corticosterone and the sensitivity of the receptor to modulation by guanyl nucleotides, properties that are typical for G protein-coupled receptors (351). Partial purification and further biochemical characterization of the roughskin newt membrane glucocorticoid receptor revealed a glycoprotein with an apparent molecular mass of 63 kDa and a pI of $\sim$5.0. These characteristics differ from classical glucocorticoid receptor and give strong evidence that these two receptor proteins are distinct (135). Ligand-binding competition studies on neuronal membranes of the roughskin newt demonstrated the potencies of a subset of kappa opioid ligands to displace $^3$H-corticosterone binding to the neuronal membranes (136). Displacement was achieved by dynorphin 1–13 amide, U 50488, naloxone, bremazocine, and ethylketocyclazocine. Kinetic analysis suggested a direct rather than an allosteric interaction with the $^3$H-corticosterone binding site. The authors conclude that their results support the hypothesis that the high-affinity membrane binding site for $^3$H-corticosterone is located on a kappa opioid-like receptor.

A very compelling comparison of the steroid binding characteristics of corticosteroid binding sites in plasma,
brain cytosol, and neuronal membranes was reported by Orchinik et al. (350). Most interestingly, the neuronal membrane preparations were very selective for corticosterone with affinities more than three orders of magnitude lower for the GR agonist dexamethasone and the antagonist RU 486. The plasma binding sites, presumably corticosteroid binding globulins, were less selective. In brain cytosol, RU 486 bound with highest affinity, followed by dexamethasone ~ corticosterone. The authors suggested that the various types of binding sites can be unambiguously identified using selectivity profiles and that rapid responses to corticosteroids in amphibian membranes are unlikely to be mediated by receptors that are either related to classic receptors or corticosteroid binding globulin.

On the other hand, and apart from the results described above, there is evidence that a specialized form of the GR is localized in the plasma membrane (165). As already mentioned above, the lysis response of S-49 mouse T-lymphoma cells to glucocorticoids has been, by means of an anti-GR antibody, attributed to a glucocorticoid receptor-like antigen that is located in the lymphoma cell membranes (164). Subsequently, membrane glucocorticoid receptors have been reported in rat liver cells (192), human leukemic cell lines, and cells from human leukemic patients (168). With the use of multiple segregation techniques, it was possible to separate different sublines of murine S-49 lymphoma and human CCRF-CEM T-leukemia lines and thus achieve populations enriched and depleted for glucocorticoid membrane receptors (167, 415). Immunoaffinity purification and Western blot analysis of membrane extracts from these membrane receptor-enriched cell lines showed a membrane GR (mGR) pattern in a molecular mass range from 42 to 150 kDa (381). The differences in size between GR (94 kDa) and mGR (150 kDa) have been suggested to be due to post-translational modifications of the membrane receptor (166). Together with mGR, the heat shock proteins hsp70 and hsp90 were coprecipitated as demonstrated by monoclonal antibodies, thus suggesting an interaction of mGR with these molecules under physiological conditions, although its role remains unclear at the moment (381).

The expression of mouse membrane GR is highly correlated with the expression of a distinct GR transcript denoted transcript 1A. The gene encoding the murine GR spans ~110 kb, and the transcripts are assembled from nine exons. There are five glucocorticoid receptor transcripts known so far (1A-1E), all differing only at their 5'-ends and generated by alternative splice mechanisms in exon 2 (92, 93, 170, 467).

Interestingly, within the 5'-untranslated region of GR transcript 1A there are five small open reading frames (ORFs), one of them encoding a peptide of ~8.5 kDa that seems to be associated with the translational regulation of GR (117). Indeed, there is some evidence for a relation in the regulation of the expression of GR and mGR. In the brain of wild-caught house sparrows it could be shown that the levels of membrane corticosteroid receptors varied seasonally, being significantly lower during the nesting season than during molting or wintering stages. Affinity of the membrane receptor was not influenced by the seasonal changes. Furthermore, the cytosolic and membrane receptor numbers were regulated in opposite directions, and thus suggesting not only different functions for membrane and intracellular receptors but also different mechanisms that regulate their expression (71).

From the findings discussed above, there is evidence for both nonclassic receptors and a membrane form of classic GR, probably originating from alternate transcription, that may mediate nongenomic responses to glucocorticoids.

2. Progesterone

As discussed in section mB3, it has been shown that progesterone nongenomically acts on oocyte maturation in Xenopus. In the past, it has been assumed that the actinomycin D-insensitive progesterone-induced maturation (456) is mediated by cell surface rather than nuclear receptors. Thus progesterone covalently attached to either BSA or polymers can induce maturation when applied to the outside of an oocyte (181, 277). However, this approach is controversial (see sect. iv). Moreover, free progesterone can induce maturation when applied to the outside of an oocyte but not when injected directly into oocytes (277, 284). With regard to this assumption, several membrane progesterone binding sites have been described (reviewed in Refs. 276, 320).

On the other hand, two recent papers reported the cloning and characterization of cDNAs for Xenopus homologs of the classic PR, termed XPR and XPR-1 (34, 482). In both papers indexes for the involvement of this homolog in progesterone-induced maturation are given. XPR-1 antisense oligonucleotides were found to inhibit progesterone-induced maturation (482), and the injection of a truncated XPR-mRNA was found to accelerate oocyte maturation (34). In both cases, the ability of XPR to promote maturation was not affected by actinomycin D (34, 482). In a further work, the role of XPR in nongenomic signaling in Xenopus oocytes was examined (14). The authors found that a fraction of one form of XPR was attached to the plasma membrane. In addition, it has been shown that progesterone causes XPR to become associated with phosphatidylinositol (PI) 3-kinase activity, and an interaction of XPR with p42 MAPK was observed (14).

Recently, a progesterin maturation inducing steroid receptor has been characterized on spotted seatrout oocytes (481). The deduced amino acid sequence of this protein shows seven putative transmembrane domains.
The protein may therefore represent a G protein-coupled membrane receptor.

In addition to the well-studied nongenomic steroid effects in sperm, the existence of corresponding membrane receptors has been examined. The first evidence supporting the presence of steroid binding sites on human spermatozoa came from a work by Hyne and Boettcher (216) where specific 17β-estradiol receptors were detected for which progesterone could compete. The hypothesis of progesterone receptors on the surface of human sperm was further substantiated by experiments done with membrane-impermeable agents like BSA-conjugated progesterone (60, 292) and progesterone-BSA-FITC (480). Many features of the sperm response to progesterone and contradictory findings with regard to the presence and the state of the protein involved suggest the presence of different types of progesterone receptors in sperm (reviewed in detail in Ref. 400).

The presence of the classic intracellular PR in spermatozoa is discussed controversially. Antibodies directed against the DNA binding domain of PR did not detect any specific band (267, 413), confirming earlier experiments that demonstrated PR not to be present in human sperm (87). In different studies where an antibody raised against the steroid binding domain of PR (c-262) was used, protein bands with molecular masses of 50–52 kDa (413) or 54 and 57 kDa were labeled (267). The application of this antibody leads to an inhibition of progesterone-induced Ca²⁺ influx and the acrosome reaction (413). However, since human PR consists of a ~94 kDa A form and a ~120 kDa B form (132), the marked proteins are noticeably smaller in size. Nevertheless, several exon-deleted or truncated PR variant mRNAs have been observed, at least in breast cancer cells (253). Recently, a PR transcript comprising the DNA binding domain as well as the hormone binding domain, has been detected in RT-PCR experiments (414).

In human sperm extracts, analogous to our findings in porcine liver microsomes (see below), proteins with molecular masses of 25–30 kDa and 50–60 kDa could be labeled by antibodies against the rat homolog of mPR (25Dx) (57). Therefore, the existence of an, at least related, progesterone membrane binding protein is very likely in sperm. For this reason, our group investigated the influence of a mPR-specific antiserum on rapid progesterone-induced Ca²⁺ fluxes in human sperm. In the presence of the IgG fraction of a specific antibody (1:10 dilution), a significantly reduced progesterone-induced Ca²⁺ increase was found (140). These data are in accordance with recent experiments done with the same mPR-specific antibody. Thereby, and by using the same antibody, an inhibition of the progesterone-initiated acrosome reaction by 62% was found (75).

Besides the reproductive system, the presence of membrane progesterone binding sites has been examined in several tissues or cells. For instance, in brain plasma membrane fractions, progesterone conjugated to radiolabeled BSA has been frequently used to identify putative steroid receptors. In the medial preoptic area-anterior hypothalamus of ovariectomized rats, high- and low-affinity progesterone binding sites have been described that are likely to be associated with G proteins (83, 84, 392, 540). Moreover, photoaffinity labeling done with a progesterone analog in mouse brain membranes identified four protein bands with molecular masses ranging from 29 to 64 kDa (76).

Progesterone and related compounds were also found to bind to GABA₆ receptors (322, 550). Interestingly, in appropriate KO mice, the absence of the γ-subunit of GABA₆ receptor leads to a decrease in sensitivity to neuroactive steroids such as pregnanolone (306).

Consistent with reports on rapid actions of progesterone in the ovary, specific membrane progesterone binding sites have been discovered in luteal membranes from various species. However, the binding of the steroid could only be demonstrated in the presence of digitonin (68, 297, 390).

In granulosa cells of immature rats, progesterone was found to inhibit apoptosis (364). It is not likely that this action is mediated through the classic nuclear PR, since this protein does not seem to be expressed in these cells (363). Alternatively, a 60-kDa membrane protein has been detected in granulosa cells by the use of an antibody directed against the steroid binding domain of nuclear PR (c-262) (365). In ligand blot and ligand binding studies, it has been shown that this protein specifically binds progesterone (363).

The progesterone metabolites 5α-pregnan-3,20-dione and 5α-hydroxy-4-pregnen-20-one, which are assumed to modulate breast cell proliferation, were found to bind specifically to the membrane fraction of MCF-7 breast cancer cells. In saturation analyses, the respective bindings sites showed dissociation constants of 4.5 and 484 nM, respectively (522).

In search of specific steroid membrane binding sites and with regard to the reports on rapid progesterone effects on liver cell conductance (504) and progesterone membrane binding sites in hepatocytes (217, 488), our group was able to characterize membrane progesterone binding sites (mPR) from porcine liver microsomes. After purification, the binding capacity corresponds to the enrichment of polypeptides of 29 to 56 kDa with the latter probably representing a dimer of the 28-kDa protein (139, 301). However, the native progesterone membrane receptor is likely to be an oligomeric protein complex of ~200 kDa composed at least in part of the 28- and 56-kDa proteins (300). Interestingly, mPR was localized to intracellular membranes (143). Because the hydrophobicity of steroids allows for rapid entrance into cells, this finding is
still compatible with the assumption that mPR may be involved in nongenomic progesterone action.

Sequence analyses demonstrated that mPR has no significant identity to any protein with currently known function, thus nor to the intracellular PR or other steroid receptors (141, 174). Expression of the mPR-cDNA in Chinese hamster ovary (CHO) cells leads to an increase in microsomal progesterone binding compared with microsomes from mock-transfected CHO cells (140). In this regard, high-affinity binding of progesterone to microsomes from intact bovine lens epithelium has been reported. Using a mPR-specific antibody, a microsomal 28-kDa protein with high homology to mPR was detected (90, 546).

Interestingly, the rat equivalent of mPR, termed 25-Dx (436), has been associated with behavioral processes. In the ventromedial hypothalamus of rats, 25-Dx expression has been shown to be repressed by progesterone after estradiol priming of ovariectomized animals. Moreover, compared with wild-type littermates, higher expression levels of 25-Dx were seen in female PR knockout mice, suggesting a scenario in which the activation of the intracellular PR represses the expression of 25-Dx (238).

A very recent addition to the candidate receptors for rapid progesterone action comes from Zhu et al. (547). A 40-kDa membrane protein was identified after screening a cDNA library from spotted seatrout ovary, possessing seven predicted transmembrane domains, which is characteristic for G proteins. Protein recombinantly expressed in *Escherichia coli* bound progesterone with high affinity ($K_D = 30 \text{ nM}$) and had significant preference for 21-carbon steroids unsubstituted at carbon 11. In a mammalian cell line transfected with the novel receptor, progestins activate a MAPK pathway. This finding together with changes in protein abundance in response to hormones in the course of oocyte development suggests that this is a novel, membrane progesterone receptor with a defined action. Other homologous genes have been demonstrated, and members of this gene family have been detected in human tissues (548).

For progesterone-induced effects, much evidence points to the existence of nonclassic receptors, particularly for the well studied induction of the acrosome reaction. Evidence for classic receptors is mainly drawn from immunoochemical studies, with the exception of *Xenopus*, where two recently published studies (14, 34; see sect. iv) convincingly demonstrate a role of the classic PR in nongenomic events.

### 3. Estrogens

Estrogens classically exert their biological effects by activation of ERs modifying gene expression. Again, ERs, which regulate the transcriptional activity of target genes by interacting with different DNA response elements, exist at least in two subtypes, ER$\alpha$ and ER$\beta$, deriving from different genes. In vitro studies suggest that both receptors may play redundant roles in estrogen signaling; however, tissue localization studies indicate different expression patterns for each receptor (198). In addition to their effects on gene transcription, ERs may be involved not only in genomic steroid action, but also in rapid nongenomic, nongenomic effects. Such nongenomic effects are initiated at the plasma membrane and are postulated to be mediated by a membrane-bound ER that seems to be very similar, if not identical, to the classical intracellular ERs.

One of the fundamental studies was done in rat pituitary tumor cell lines. Antibodies raised against epitopes of ER stained membrane proteins of immuno-selected GH$_4$/B6 cells (354). Moreover, with the use of several antibodies to ER$\alpha$, the membrane version of ER$\alpha$ could be detected making it likely that the membrane and nuclear proteins are highly related. Estrogen at very low concentrations induces prolactin release from GH$_4$/B6 cells within minutes of application, and the prolactin release is also elicited or inhibited by ER$\alpha$-specific antibodies (512). Similar results could be obtained in CHO cells transfected with both of the receptors subtypes: small amounts of both ER$\alpha$ and ER$\beta$ were expressed at the plasma membrane (394).

The evidence of a membrane localization of the subtype ER$\alpha$ could be confirmed in cultured hippocampal neurons. Antibodies directed against ER$\alpha$ showed positive membrane staining in nonpermeabilized neurons. On the other hand, in permeabilized hippocampal neurons, the labeling for ER$\alpha$ could be detected in the perinuclear area, but abundant staining for ER$\beta$ was found throughout the cell including the neurites. Moreover, the immunoreactivity of ER$\alpha$ was reduced throughout the neurons in the presence of antisense oligonucleotide directed against the translation start site of ER$\alpha$. With the use of conventional and confocal microscopy, most of the antigen could be localized in the extranuclear compartment (103).

Activation of the endothelial nitric oxide synthase (eNOS) leading to increased levels of nitric oxide (NO) is a fundamental determinant of cardiovascular homeostasis. As previously shown, 17$\beta$-estradiol rapidly stimulates the eNOS of cultured endothelial cells, which can be completely blocked by the classical ER antagonists tamoxifen and ICI 182780 (88, 245). Moreover, the stimulation of eNOS by 17$\beta$-estradiol was further enhanced by overexpression of ER$\alpha$ (443). The involvement of ER$\alpha$s in the nongenomic actions of estrogens was confirmed by the demonstration that the rapid response of eNOS to 17$\beta$-estradiol could be detected in COS-7 cells transfected with the wild-type ER$\alpha$ and eNOS, but not by transfection with eNOS alone. Again, inhibitors of Ca$^{2+}$ influx, tyrosine...
kinases, or MAPK inhibited the stimulation of eNOS by 17β-estradiol (442). Similar results were obtained in human umbilical vein endothelial cells: 17β-estradiol and the 17β-estradiol-BSA-conjugate activated MAPK as well as cGMP synthesis and NO release, whereas ICI 182780 blocked these effects (412).

In bovine aortic endothelial cells (BAEC), 17β-estradiol rapidly increases cGMP release; this effect is sensitive to the ER antagonist ICI 164384 (182). In the same cell system, NO synthesis is augmented rapidly by both 17β-estradiol and BSA-17β-estradiol-conjugate, and the ER antagonist ICI 182780 completely blocked estrogen-stimulated NO release, which supports the assumption that a plasma membrane receptor similar to ER is involved in these effects (231).

Results of a recently published investigation indicate that G proteins could be involved in the signaling of the nongenomic actions of 17β-estradiol (Fig. 5). 17β-Estradiol induced increases of NO activity in intact endothelial cells that were fully blocked by the G protein inhibitor pertussis toxin. Coimmunoprecipitation investigations of the plasma membrane of COS-7 cells transfected with ER and specific Go proteins demonstrated that 17β-estradiol induced interactions between ERs and Go. The observed ER Go interaction was inhibited by ICI 182780 and pertussis toxin. Cotransfection of Go into COS-7 cells expressing ER and eNOS led to a pronounced increase in 17β-estradiol-mediated eNOS stimulation (534).

Further insights in the molecular mechanisms involved in the activation of eNOS could be obtained in a human endothelial cell hybridoma line. 17β-Estradiol rapidly induced phosphorylation and activation of eNOS through the PI 3-kinase-Akt pathway. One of the downstream targets of the PI 3-kinase pathway is the serine/threonine kinase Akt, also referred to as protein kinase B (PKB). Moreover, the 17β-estradiol-induced NO release could be blocked by the PI 3-kinase inhibitor LY 294002, and 17β-estradiol and the BSA-17β-estradiol-conjugate both increased the phosphorylation of Akt and eNOS. The functional involvement of Akt could be confirmed using adenoviral approaches, since a dominant-negative Akt abolished the 17β-estradiol-stimulated NO release (208).

Other findings that point to the modulation of a G protein-coupled receptor by an ER have been reported earlier (243). Here, estradiol attenuated the hyperpolarizing action of μ-opioids on hypothalamic neurons. Interestingly, both the ER agonist diethylstilbestrol and the antagonist ICI 164384 blocked the estradiol effect. Because the response could be mimicked by protein kinase A (PKA) activators and blocked by the corresponding inhibitors, PKA is apparently involved. More detailed studies revealed the involvement of PKC, that may act directly to uncouple GABA receptors from K+ channels, or on adenyl cyclase and thus indirectly on PKA, uncoupling the μ-opioid receptors from the ion channels (229).

Many of the findings on MAPK activation by estrogens were obtained in tissues containing both ERα and ERβ. To individually explore the role of each ER in the rapid activation of the MAPK signaling pathway, ER-negative RAT-2 fibroblasts were transfected with DNA clones encoding either ERα or ERβ. For both receptors, 17β-estradiol induced a rapid phosphorylation of MAPK. Differences were found for the time course of activation: after activation, MAPK dropped within 15 min to control levels in ERα cells, whereas in ERβ-transfected cells, MAPK remained activated for at least 1 h. This may be important, since the timing and duration of MAPK activity seems to be essential for nerve growth factor (NGF)-
induced cellular differentiation. Moreover, the activation of MAPK in ERα transfected cells could be partially or completely blocked by tamoxifen and ICI 182780. In contrast, in ERβ-transfected cells both compounds were less sensitive to inhibit that effect (502).

In addition to its function in the female reproductive system, 17β-estradiol plays an important role also in the development and regulation of the male reproductive system. Results of ligand blot analysis indicate a specific estradiol binding protein in human sperm. The same protein band could be found by an antibody directed against the steroid binding domain of the classic ER (αH222). The binding of 17β-estradiol was correlated with a rapid and sustained increase of [Ca2+], These effects on [Ca2+], could also be obtained by the use of the BSA-17β-estradiol-conjugate, which is incapable to penetrate the plasma membrane (18). Furthermore, the receptor seems to be involved in the activation of two different signal transduction pathways. In addition to the increase of [Ca2+]i, 17β-estradiol stimulated tyrosine phosphorylation of several sperm proteins, resulting in inhibition of progesterone-induced calcium influx and acrosome reaction (268). The data indicate that 17β-estradiol might be able to modulate the nongenomic effects of progesterone in human sperm during the fertilization. The effects of progesterone as a well-known stimulus for human spermatozoa are described in detail in section IVB.

As mentioned above, nongenomic estrogen effects seem to be mediated by a membrane-bound ER similar to the classical intracellular ER. Interestingly, some studies indicate binding sites which could be different from ER. Some years ago, it could be demonstrated that 17β-estradiol leads within few minutes to a rapid and sustained (2 h) tyrosine phosphorylation and activates MAPKs as well as ERK1 and ERK2 in PC12 cells. These effects can be blocked by the MAPK/ERK1 inhibitor PD 98059, but not by the classic ER antagonist ICI 182780. Moreover, the ability of estradiol to phosphorylate ERK persisted in ERα KO mice (483). Up to now, it remains unclear if these effects of estrogen on ERK activation are mediated by the activation of ERβ or by an yet unidentified ER. 17β-Estradiol can potentiate kainate-induced currents in isolated hippocampal neurons of the ERα KO mouse or wild-type mice. The preservation of these rapid 17β-estradiol actions in presence of ICI 182780, which blocks both ERα and ERβ, suggests the existence of a functional membrane ER that is distinct from the intracellular nuclear form (502). Further evidence could be obtained recently in the mouse macrophage cell line IC-21. 17β-Estradiol signaling as measured by a rapid increase in [Ca2+]i, was initiated at the plasma membrane: the plasma membrane-impermeable 17β-estradiol-BSA-conjugate also induced this rise in [Ca2+]i. Again, this rise in [Ca2+]i, could be inhibited by pertussis toxin, and the phospholipase C inhibitor U 73122 blocked the release of intracellular Ca2+, indicating that the membrane receptor of 17β-estradiol belongs to membrane receptors that are coupled to phospholipase C via a pertussis toxin-sensitive G protein. Moreover, the ER blockers tamoxifen and raloxifene did not inhibit the rapid increase of [Ca2+]i of IC-21 cells induced by both 17β-estradiol and its BSA-conjugate. The membrane receptor properties of 17β-estradiol found in IC-21 cells seem to be similar to other G protein-coupled receptors including sequestration after ligand binding. Also, the particular receptor becomes sequestered within minutes after binding of 17β-estradiol. The reason for the 17β-estradiol receptor internalization remains unknown (47).

Moreover, the assumption that many of the effects of 17β-estradiol involve membrane-associated mechanisms independent of classical ERs has recently been revisited. It was demonstrated that 17β-estradiol-BSA-conjugate induced time-dependent increases in PKC in resting zone (RC) and growth zone chondrocytes (GC) from female rat costochondral cartilage within minutes. These effects could be prevented in the presence of the G protein inhibitor guanosine 5′-O-(2-thiodiphosphate) (GDPβS) in both cell lines, whereas the G protein activator guanosine 5′-O-(3-thiotriophosphate) (GTPγS) increased PKC in 17β-estradiol-BSA-conjugate incubated GC cells but had no effect in RC cells. In both RC and GC cells, the phospholipase C (PLC) inhibitor U 73122 blocked 17β-estradiol-BSA-conjugate stimulated PKC activity, whereas the phospholipase D (PLD) inhibitor wortmannin had no effect. Neither the classical ER antagonist ICI 182780 nor the agonist diethylstilbestrol had any inhibitory or stimulatory effects. The nongenomic mechanism of the 17β-estradiol-BSA conjugate seems to be dependently mediated by G protein-coupled PLC (474).

For many of the nongenomic estradiol effects, evidence suggests the participation of either classic ER itself or a closely related form located at the membrane. However, receptors seem to exist that extensively differ in their properties and are unlikely to be related to ER.

4. Androgens

In the past 25 years, membrane androgen binding sites that are discussed to be responsible for rapid androgen signaling have been described in several tissues or cells (for review, see Ref. 144). But of late, the nature of these binding sites has been controversial. Recently, binding sites for testosterone on the surface of T cells and IC-21 macrophages have been detected with testosterone-BSA-FTTC that are likely to be involved in the context of nongenomic testosterone action on [Ca2+]i (44, 46). However, in IC-21 macrophages, the absence of the classic AR has been demonstrated even by independent experimental approaches. Incubation of the cells with an anti-AR antibody (directed against the NH2 terminus) did not significantly label the cells, and in West-
ern blot analyses using this antibody no immunoreactive band was found. In addition, RT-PCR approaches using primers spanning the DNA-binding domain and three different regions from the COOH terminus of the AR-cDNA did not reveal the expected PCR products, whereas the correct amplificates were found in the control RNA from mouse testes (46). Moreover, in T cells where rapid androgen signaling has been shown (see also sect. uC3), anti-AR antibodies directed against the NH2 or COOH terminus of AR did not reveal any significant fluorescence on the cell surface. However, in contrast to the common view that the classic AR is absent in these cells (348), an inactive form (with regard to genomic events) of AR was demonstrated in the cytoplasm of T cells (44).

On the other hand, a participation of the classic AR is discussed in the dihydrotestosterone-induced rapid activation of MAPK. Only PC3 cells transfected with AR-cDNA showed this effect; cells transfected with the sham plasmid were inactive (370).

Moreover, the sex hormone binding globulin (SHBG) is considered to play a role in permitting certain steroids to act without entering the cell (405). Upon ligand binding, the SHBG interacts with a high-affinity membrane receptor. This SHBG-receptor complex has been described to cause a rapid generation of cAMP after exposure to the respective steroid. For example, in prostate cell membranes, an increase in intracellular cAMP in response to both androgens and estradiol, via binding to a membrane SHBG receptor, has been shown (120, 405). For androgens, evidence is mixed, pointing to either classic AR or a nonclassic receptor, or even participation of additional proteins.

5. Neurosteroids

Many adrenal and gonadal steroids as well as some of their synthetic derivatives can modulate neuron excitability and synaptic communication in the central nervous system. For these compounds the term neuroactive steroids has been coined (274) or neurosteroids for those which mainly glial cells can synthesize de novo (31). These are traditional definitions, under which most steroids discussed in other sections would be covered as well. However, this section focuses on phenomena that are tightly linked to receptors occurring mainly in the central nervous system. Neuroactive steroids engage the “classical” receptors of progesterone and androgens regulating transcription through interaction with these intracellular receptors. However, nongenomic ways of action are known for a variety of neurosteroid effects that involve other than the classical steroid receptors. Neuroactive steroids may modulate an array of neurotransmitter receptors, G protein-coupled receptors, and ligand-gated ion channels are the GABA_A, the NMDA, the Sigma1, the 5-HT_3A, and the glycine receptors.

Just as diverse as the array of receptors and compounds involved in neurosteroid action, are the physiological effects they exert. Neuroactive steroids influence sleep patterns, the reaction to stress, sex recognition, memory function, brain plasticity, vigilance, and mood (33, 410). Hence, they may have a therapeutic potential far beyond today’s applications.

A) GABA_A Receptor. Due to their specific potential to allosterically enhance or block the action of GABA or other GABAergic substances like barbiturates, and benzodiazepines by interaction with the GABA_A receptor, neuroactive steroids may exert extremely diverse neuropsychological effects.

Depending on the specific structure of the GABA_A receptor with its subunits forming ligand-gated ion channels, neuroactive steroids can alter the excitability of GABAergic neurons (523). GABA receptors are heteroligomeric proteins containing a number of allosterically interacting binding sites. The neurotransmitter GABA as well as benzodiazepines, or barbiturates, can bind to these structures (62, 454). 3α,5α-Tetrahydroprogesterone (3α,5α-THP) and 3α,5α-tetrahydrodeoxycorticosterone (3α,5α-THDOC) were the first steroids that have been shown to modulate neuronal excitability by interaction with GABA_A receptors (274). As potent barbiturate-like ligands of the GABA_A receptor-chloride ion channel complex, both steroids inhibit binding of the convulsant t-butylbicyclophosphorothionate (TBPS) to the GABA receptor complex at concentrations between 100 nM and 10 μM. As coagonists at the GABA_A receptor, they also increase the binding of flunitrazepam (169). In addition, the GABA_A receptor is involved in 3α,5α-THP- and 3α,5α-THDOC-stimulated chloride uptake into isolated brain vesicles, as well as neurosteroid-potentiated inhibitory actions in cultured rat hippocampal and spinal cord neurons (386). The pharmacological activity of benzodiazepines differs with the α-subunit composition and necessitates the presence of a γ-subunit of the GABA receptor. In contrast, the effects of neuroactive steroids do not depend on such strictly defined basic requirements for their structure-activity relationship. The potentiating and direct actions of the steroids used by Puia et al. (385) were expressed with every combination of subunits tested. Recent studies suggest that anabolic steroids may induce subunit-specific rapid modulation of GABA_A receptor-dependent mediated currents in brain regions essential for neuroendocrine function (223).

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optic area (mPOA). These two brain regions are critical for the expression of reproductive behavior. In doses compatible with steroid abuse, all three steroids significantly enhanced peak synaptic current amplitudes and prolonged synaptic current decays in neurons of the VMN. In contrast, peak current amplitudes of synaptic currents from neurons of the mPOA were significantly diminished, suggesting a GABA_A receptor subunit- and brain region-specific alteration of neuroendocrine function mediated by neuroactive anabolic steroids (223). This finding substantiates other in vitro investigations examining GABA_A receptor binding by steroids. The blockade of GABA_A receptors by dehydroepiandrosterone sulfate (DHEAS) was found to be different in the posterior and the intermediate pituitary, and in the latter even varied from cell to cell (201). The steroid may thus have a role in neuropeptide secretion, and the differing properties of GABA receptors may participate in the regulation of peptidergic systems. However, these results were obtained in a rat model with concentrations of circulating DHEAS several orders of magnitude lower than in humans. With the use of recombinant GABA_A receptors, expressed in an insect cell line, enhancement by steroids of [35S]TBPS binding was sensitive to the presence of the γ_2-subunit and the characteristic composition of the α-subunit (α_1 β_2 γ_2S > α_1 β_2, α_3 β_3, α_6 β_6, γ_2S, and α_6 β_6 δ). Addition of RU 5135, a GABA antagonist, reduced the inhibitory phase and exposed a small enhancement of TBPS binding. The subunit-dependent interactions of steroid and GABA site ligands are in line with a three-state model in which the receptor having bound one molecule of GABA or the respective steroid has a different affinity for TBPS than the resting state, while the receptor occupied by two molecules of GABA, steroid, or both has little affinity for TBPS (463). Corticosterone, which is elevated under stress conditions, has been found to decrease the negative modulation of TBPS binding in hippocampus by both GABA and 3α,5α-thioldihydroxyprogesterone, while augmenting the enhancement of flunitrazepam binding by these steroids in the dentate gyrus (349). In addition, striking regional differences in the modulation of TBPS binding were found after prolonged corticosterone treatment.

Neuroactive steroids may also alter GABA_A receptor subunit composition per se. Withdrawal from 3α,5α-THP was associated with an upregulation of the α_1-subunit of the GABA_A receptor in rat hippocampal slices. This was functionally linked to increased anxiety and benzodiazepine insensitivity (196, 458). 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 (106). If these findings can be substantiated, a concept embracing nongenomic as well as genomic mechanisms may be developed by which steroids alter brain function.

On the other side, structure prerequisites for steroids have indeed been characterized as essential for a steroid-induced positive allosteric interaction at the GABA_A receptor. Only those steroids containing a 3α-OH-group within the A-ring of its molecule are apparently able to enhance GABA or benzodiazepine action at the GABA_A receptor (171, 362). All steroids with a 3β-OH conformation that have been investigated so far have been ineffective in increasing GABA_A receptor-mediated Cl^- conductance or flux (362, 386). These results harbor speculations that 3α-hydroxysteroids have a distinct stereoselectivity at the GABA_A receptors. Analogously, 3α-reduced pregnane steroids (e.g., DHEA-S, PREG-S) have been shown to exert GABA-antagonistic effects at the GABA_A receptor (244, 409, 445). In the search for synthetic steroids that effectively enhance GABA action at the GABA_A receptor, Anderson and co-workers (6) refined this aspect. Steroid in vitro and in vivo activities similar to 3α-hydroxyprogesterone all had an ether oxygen on the β-face of the steroid D-ring. This suggests that for effective coagonism at the GABA_A receptor, the hydrogen bond-accepting substituent should be near perpendicular to the plane of the ring on the β-face of the steroid (6). Recent work has identified the structural basis for steroids augmenting GABA_A receptor function while inhibiting NMDA neurotransmission (296). The combination of these properties would make a clinically useful anesthetic steroid.

Much effort has been spent on characterizing a specific steroid binding site on the GABA_A receptor. Today, there is considerable evidence that steroid recognition sites reside on the GABA_A receptor, and not in the bilayer surrounding it. In steroid-insensitive Xenopus oocytes a temporary expression of GABA_A receptor subunits caused steroid responsiveness (449) even though desensitization did not show rigorous stereoselectivity (528). In addition, 3α,5α-dihydroprogesterone has been shown to modulate ligand binding to solubilized GABA_A receptors in a fashion compatible with membrane-bound receptors (179).

The coagonism as well as the allosteric antagonism of neurosteroids to GABA at the GABA_A receptor mediate multiple functional effects. These are briefly discussed below but have been extensively reviewed elsewhere (244, 362, 411, 509).

b) NMDA receptor. The N-methyl-D-aspartate (NMDA) class of glutamate receptors (NMDAR) are essential for learning, memory, and development in the central nervous system (317, 428). NMDARs are multimeric complexes formed from both NMDA receptor subunit (NR1) and modulatory NR2 subunits (316, 328).

The subunit composition is thought to be essential for receptor functionality (154) and its pharmacological characteristics, e.g., single-channel conductance and deactivation kinetics (147, 466) as well as sensitivity to Mg^2+ (308), Zn^2+, (213) and ifenprodil (525).

Subunit composition of the NMDAR has also been proposed as critical for the effects of neuroactive ste-
The modulatory action of steroids on NMDA receptors and excitatory postsynaptic transmission was studied in outside-out patches of rat motoneurons. NMDA-induced responses were significantly enhanced by 20-oxopregn-5-en-3β-yl sulfate (PS), while in the presence of 20-oxo-5α-pregn-3α-yl sulfate (3α,5α S) and 20-oxo-5β-pregn-3α-yl sulfate (3α,5βS) they were significantly diminished. PS and 3α,5βS both altered the relative distribution of the openings to individual conductance levels. In controls, the most frequent openings of the NMDAR channels were at the 70-pS conductance level, whereas in the presence of PS or 3α,5βS, the most frequent openings were at the 55-pS conductance level (1). These actions of neuroactive steroids are most likely mediated nongenomically due to their rapid onset of action which does not allow for the classic way of action of steroids via intracellular steroid receptors.

Pregnenolone, a rather well investigated neuroactive steroid, has been shown to enhance memory performance in various test paradigms in rodents (285). This effect has been linked to a nongenomic allosteric modulation of the NMDAR (304). The hypothesis of nongenomically mediated effects of pregnenolone at the NMDA is corroborated by findings that pregnenolone sulfate blocks learning and memory impairment induced by d-2-amino-5-phosphonovalerate, a competitive NMDA receptor antagonist (286). Furthermore, pregnenolone sulfate has also been shown to act as a coagonist at the NMDA receptor, allosterically modulating receptor currents within seconds (65).

Apart from these well-characterized nongenomic effects at postsynaptic receptors, pregnenolone sulfate has also been shown to act at glutamatergic synapses in the CA1 region of the hippocampus of rats. It enhanced paired-pulse facilitation of glutamate excitatory postsynaptic potentials on ionotropic NMDA receptors with an EC_{50} <1 μM (358). Similarly, Monnet et al. (310) demonstrated that pregnenolone sulfate enhances NMDAR-dependent norepinephrine release in hippocampal slices via the sigma1-receptor (310). These results point to a further site of action in addition to postsynaptic receptors at which neurosteroids may have an effect on nervous system plasticity (see sect. dD5c).

Contrasting the fast, almost instantaneous nongenomic effects of neuroactive steroids, these compounds have also been shown to act via the classical intracellular steroid receptor cascade, e.g., influencing subunit composition of NMDAR. High doses (5 and 15 mg/kg) of nandrolone decanoate administered over 14 days intramuscularly produced a significant decrease in the mRNA expression of the NR2A receptor subunit both in the hypothalamus and hippocampus of male rat brain (249). A decrease in the level of NR2B receptor mRNA was observed in hypothalamus at the lower dose of nandrolone.

C) SIGMA1 RECEPTOR. The sigma1 (σ1)-receptor seems to be distinctly different from any other known transmitter receptor. It embodies a unique binding site in the central nervous system and peripheral organs. Nevertheless, distribution within the central nervous system and molecular structure have not been characterized satisfactorily. After the σ1-receptor protein was purified and its cDNA cloned in several species (200, 228, 440), specific regions involved in σ1-receptor function, analyzed by expression patterns of receptor mRNA in mice brain, were detected. Substantial mRNA expression was found in cerebral cortex, hippocampus, and Purkinje cells of the cerebellum (441). Ligands of the σ1-receptor may exert a potent neuromodulation on excitatory neurotransmitter systems, including the noradrenergic, glutamatergic, and cholinergic systems (49, 184, 185, 287, 309). It has been proposed that neurosteroids modulate σ-receptor activity by a G protein coupling at the neurosteroid binding site (491). DHEAS stimulated the [35S]GTPγS binding in synaptic membranes of mouse neurons. This effect was blocked by NE-100, an experimental σ-receptor antagonist. The DHEAS-induced stimulation was blocked by pertussis toxin (PTX) treatment and completely recovered by reconstitution of PTX-treated membranes with recombinant G_{t} (491). Another possible mode of action of steroids at the σ-receptor is a stimulation of Ca^{2+} influx. However, this has not been shown for central nervous system-located σ-receptors but in sperm. Prostaglandin E_{1} and progesterone induced Ca^{2+} entry and acrosome reaction, an effect that was blocked by the steroid σ-receptor ligand RU 1968 (421). It remains to be determined if these findings can be reproduced in neuronal σ-receptors.

Neuroactive steroids have the potential to inhibit the binding of central nervous system-bound σ1-receptor radioligands in vitro and in vivo. This seems to imply a direct communication between neuroactive steroids and σ1-receptors (289).

Interestingly, the progesterone-binding membrane protein isolated from liver (cf. sect. uD2) shares some properties with σ-receptors (302).

D) 5-HT_{3} RECEPTOR. Neuronal receptors of the 5-HT_{3} type are members of the Cys loop family of ligand-gated ion channels. This family of receptors also includes the GABA_{A} receptors, the glycine, and nicotinic acetylcholine receptors. All of these receptors are pentamers, usually formed by the coassembly of one to four different subunits (281). Evidence suggests that the Cys loop family of receptors is modular in design, with the extracellular NH_{2}-terminal domain containing the ligand binding site and the transmembrane regions containing the pore (128). Recent data substantiate the hypothesis that there is a high degree of structural and functional homology between receptors in the Cys loop receptor family (397). Consequently, the gonadal steroids, 17β-estradiol and progesterone, have been described as functional antagonists not only at the above-mentioned receptors but also at the 5-HT_{3} receptor.
This has been demonstrated in cells stably expressing the 5-HT₃ receptor using whole cell voltage-clamp recordings. In contrast to pregnenolone sulfate and cholesterol, functional antagonistic characteristics at the 5-HT₃ receptor have also been shown for 17α-estradiol, 17α-ethinyl-17β-estradiol, testosterone, and allopregnanolone (523). From comparative studies using various alcohols, it has been proposed that the modulation of 5-HT₃ receptor function by steroids is dependent on their respective molecular specificity. Steroid-induced antagonistic effects at the 5-HT₃ receptor are not mediated via the serotonin binding site. This has been suggested because the investigated steroids did not alter the binding affinity of [³H]GR 65630 to the 5-HT₃ receptor. In addition, kinetic experiments were able to demonstrate a different response pattern to 17β-estradiol when compared with the competitive antagonist metoclopramide (523). Gonadal steroids have been suggested to interact allosterically with the 5-HT₃ receptor at the receptor-membrane interface. This has been shown for progesterone (533). A noncompetitive, voltage- and agonist-independent mechanism that was distinct from that of open-channel blockers has also been proposed for progesterone-induced inhibition of the 5-HT₃ response (533).

Furthermore, it has been demonstrated that neuroactive steroids may have effects at both the 5-HT₃ receptor channel and adjacent voltage-gated sodium channels. The relationship between most of the compounds’ lipophilicity and their cation channel inhibiting properties is compatible with the mechanistic principle of steroid-induced inhibition of the two channels, i.e., a nonspecific hydrophobic interaction with certain membrane lipids in the neighborhood of the two channels (26). However, despite a considerable body of evidence, a satisfactory picture of neuroactive steroids action at the 5-HT₃ receptor remains elusive. A concept integrating noncompetitive and competitive nongenomic effects of neuroactive steroids and effects that may be mediated via classical intracellular steroid receptors in various types of neuronal cells has still got to be developed. Such a concept should also incorporate the interaction with other neurotransmitter receptors and feedback mechanisms regulating receptor density and subtype composition as well as concentration levels of circulating and tissue-bound steroids.

E) GLYCINE RECEPTOR. The glycine receptors belong to the same superfamily of transmitter-gated ion channels as the GABA_A receptor (240, 459). Analogously to nicotinic acetylcholine, GABA_A and serotonin 5-HT₃ receptors, the glycine receptor consists of five subunits each with four transmembrane segments (189). Two types of glycine receptor subunits have been cloned so far (α₁-α₄ and β; also see detailed review by Harvey et al., Ref. 206). The functional properties of the inhibitory receptors are likely to be heterogeneous and may be brain region or even neuron specific (377).

Neuroactive steroids have been shown to modulate glycine receptors, but data on effective concentrations are sparse. Potencies have been published for pregnenolone sulfate and progesterone in chick embryonic spinal cord (531, 532) and Xenopus laevis oocytes (275). Malsay et al. (275) also report on effects of DHEAS. The mechanism by which pregnenolone sulfate and progesterone bind to the receptor and mediate their respective effect do not seem to be identical. Progesterone exerted incomplete and noncompetitive inhibition of glycine receptor currents, whereas data suggest that pregnenolone sulfate inhibited glycine-induced receptor currents fully and competitively (531, 532). This effect has been shown to be dose dependent. Antagonism of the glycine response by pregnenolone sulfate was neither voltage nor agonist dependent, suggesting that pregnenolone does not act as an open-channel blocker. In addition, inhibition of glycine-induced currents by pregnenolone sulfate appears to be mediated competitively. The steroid brings about a parallel, rightward shift of the glycine dose-response curve (531). In contrast, the neuroactive steroid 5α-pregn-3α-ol-20-one had no effect on glycine-invoked currents in Xenopus oocytes expressing human recombinant glycine receptors (377). This heterogeneity of effects was also seen in experiments with other neurosteroids. In addition, structure-activity requirements of glycine receptors have been suggested to be different from GABA_A receptors with inhibition dominating over allosteric enhancement (176, 382). This proposal has been corroborated by findings investigating structure-activity requirements of androstane steroids, used to address the role of chirality at the C-3 position, and pregnene steroids and their respective 3α and 3β substituents. Neuroactive steroids do seem to possess stereoselectivity at carbon 3. On the other hand, strict structural requirements of the 3-substituents for potentiation versus inhibition of glycine receptor chloride channel function were demonstrated (275). It has been hypothesized that these data advocate a role for neuroactive steroids in neuronal development, since the subunit-specific effects are particularly pronounced in receptors that most closely resemble perinatal glycine receptor composition (275).

F) OTHER MECHANISMS. Both the μ-opioid and the GABA_B receptors in hypothalamic neurons have been shown to be uncoupled from K⁺ channels by estradiol. This action, however, does not proceed as allosteric interaction at the receptors directly, but requires a signaling cascade comprising PKA and PKC (229). In a similar fashion, estradiol or its membrane-impermeant derivative coupled to BSA potentiated kainate-induced currents in hippocampal neurons (195) by modulation of non-NMDA glutamate receptors. The steroid action is attributed to a G₄ protein-coupled receptor and cAMP-dependent phosphorylation in the cytosol (194, 195). Remarkably, the
impermeant derivative needed simultaneous application to both sides of the plasma membrane to be active.

Estradiol also acts directly on the striatum of ovariectomized rats to enhance dopamine release, e.g., induced by amphetamine (40, 41). Furthermore, the steroid inhibits L-type Ca$$^{2+}$$ channels within seconds in striatal neurons when present at picomolar concentrations, an effect probably mediated by a G protein-coupled receptor (298).

The neuronal nicotinic acetylcholine receptor(s) has been shown to be targets of steroids as well (355). Blockade of this receptor occurred with the synthetic steroid 3a-hydroxy-5a-androstane-17β-carbonitrile at 1.5 μM. The inhibition exhibited little selectivity for stereoisomer at the 3 and 5 positions, which is in contrast to its importance for GABA$$\alpha$$ receptor modulation. The potency to block nicotinic receptors correlated with their ability to produce anesthesia in Xenopus.

For neurosteroids, little controversy exists about the nature of receptors. Many of the neurotransmitter receptors described above are well known, unrelated to classic steroid receptors, and susceptible to modulation by steroids.

6. Mineralocorticoids

In addition to the evidence presented from KO experiments and pharmacology, some limited information about receptors for nongenomic aldosterone action is available. Specific aldosterone binding has been demonstrated using $${^3H}$$-labeled aldosterone derivatives in human mononuclear lymphocytes (HML) (9) and later in plasma membrane fractions thereof, in pig kidney and liver, as well as in microsomes from pig liver (99, 299, 518). Radioligand binding to plasma membranes was saturable with a $$K_D$$ of ~0.1 nM as confirmed by displacement experiments with unlabeled aldosterone. While canrenone and cortisol could not displace the ligand up to micromolar concentrations, fluorocortisone and desoxycorticosterone exhibited intermediate affinity. Thus aldosterone binding to HML membranes is in good agreement with respect to kinetics and steroid selectivity with functional data on rapid effects such as ion transport and second messengers, which therefore may be mediated by these sites.

Membrane binding sites from microsomal preparations of pig liver have been studied and revealed a maximum binding capacity of ~700 fmol/mg protein (299). Binding of tritiated aldosterone was saturable with two apparent dissociation constants of <11 and 118 nM, respectively. Further purification has not been achieved due to the low stability of the protein in its solubilized form. Purification from other sources such as HML has not been possible yet due to low abundance.

A different mechanism that may lead to nongenomic actions of aldosterone in epithelia has been reported from the laboratory of Brian Harvey. The α-isoform, but not the δ-, ε-, and ζ-isofoms of PKC, has been found to be directly activated by aldosterone in vitro (204). The activation was significant at 10 pM, a concentration which is within the physiological range.

Evidence for an aldosterone receptor system with properties markedly different from the classic MR may also be drawn from studies in the isolated working rat heart (29). In this system, perfusion with aldosterone at nanomolar concentration has an immediate positive inotropic effect, which is also seen with spironolactone applied instead of aldosterone. The most striking finding, however, was the inability of spironolactone to blunt aldosterone action when perfused simultaneously; instead, an additive inotropic action of both agents was found. This strongly indicates that the nongenomic action of aldosterone, and the action of spironolactone, are mediated by independent receptors.

Mineralocorticoid action probably is the topic with the most active controversy about receptors. Although some aldosterone-induced nongenomic responses share properties with genomic action of classic MR, other properties in the same systems render MR participation rather unlikely (see sect. ιC2b). Most of nongenomic mineralocorticoid action thus is probably mediated by nonclassic receptors.

7. Vitamin D$_3$

After rapid effects of vitamin D$_3$ have been demonstrated for many years, membrane binding sites potentially transmitting these effects have been subsequently described in chick intestine (329, 330) and in ROS 24/1 cells (20, 22). In chick intestine, studies with analogs of vitamin D$_3$ [particularly 1,25(OH)$_2$-7-dehydrocholesterol and 1,25(OH)$_2$-lumisterol] have provided convincing correlations between binding to the solubilized membrane receptor and the ability to initiate transcalcitria.

Larsson et al. (247) tested the ability of the vitamin D metabolite 24R,25(OH)$_2$D$_3$ to specifically bind to basal lateral membranes isolated from intestinal epithelium of Atlantic cod (a seawater fish), carp (a freshwater fish), and chicken. Specific saturable binding was shown in membranes from all three species. In addition, in isolated Atlantic cod and carp enterocytes, 24R,25(OH)$_2$D$_3$ but not 24S,25(OH)$_2$D$_3$, suppressed Ca$$^{2+}$$ uptake into cells in a dose-dependent manner, suggesting that their binding molecule(s) is receptor-like mediating rapid, nongenomic responses in intestinal cells (247).

In competition experiments, both 1α,25(OH)$_2$D$_3$ and 1β,25(OH)$_2$D$_3$ displaced [3H]-1α,25(OH)$_2$D$_3$ from membranes while 25(OH)D$_3$ did not. The $$K_D$$ value was 0.8 μM for 1α,25(OH)$_2$D$_3$ and 0.5 μM for the 1β epimer. These binding sites perfectly match with rapid effects of vitamin
D₃ regarding their selectivity for vitamin D₃ analogs, mirroring the activity of these compounds in rapid effects on electrolyte transport.

In this context, a 1α,25(OH)₂D₃ binding site located in the basolateral membrane of vitamin D-replete chick intestinal epithelium has been described that was functionally correlated with transcaltachia. This protein exhibited saturable binding for [³H]-labeled 1α,25(OH)₂D₃ (Kᵦ = 0.72 nM, B_max = 0.24 pmol/mg protein) (330). A functional correlation between the 1α,25(OH)₂D₃ membrane binding site and transcaltachia was observed in three experimental situations: 1) vitamin D deficiency, which suppresses transcaltachia, resulted in reduced specific binding of [³H]-labeled 1α,25(OH)₂D₃ to the basolateral membrane relative to corresponding fractions from vitamin D-replete chicks; 2) the 1α,25(OH)₂D₃ membrane binding site exhibited downregulation of specific [³H]-labeled 1α,25(OH)₂D₃ binding following exposure to the nonradioactive ligand; and 3) the relative potencies of two 6-s-cis-analogs of 1α,25(OH)₂D₃ [1α,25-(OH)₂7-dehydrocholesterol and 1α,25-(OH)₂-lumisterol] to bind to the 1α,25(OH)₂D₃ membrane protein and their ability to initiate transcaltachia were congruent (329). Further experiments done with basolateral membranes of vitamin D-replete chick intestinal epithelium showed that 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 (332). A protein of similar size was also labeled by the affinity ligand [¹⁴C]-1α,25(OH)₂D₃-bromoacetate. The labeling was reduced in the presence of an excess of unlabeled secosteroid. In contrast, the nuclear VDR could not be detected in these basoalteral membrane fractions by a monoclonal antibody against the nuclear VDR (9A7) in Western blot analyses (332). The same affinity reagent was found to label a membrane protein in ROS24/1 cells that was identified to be annexin II (21). In addition, antibodies to annexin II blocked the vitamin D-induced increases in [Ca²⁺]ᵢ and diminished the binding of 1α,25(OH)₂D₃ to the protein in partially purified plasma membranes. However, these findings still await confirmation. An antibody directed against a chick 1,25(OH)₂D₃ binding protein identified a 66-kDa protein in rat chondrocytes (331) and also blocked the 1α,25(OH)₂D₃-dependent increase in PKC activity, supporting the hypothesis that a membrane receptor is involved in the initiation of rapid nongenomic effects.

Interestingly, a rapid increase of [Ca²⁺]ᵢ has also been described in the osteoblastic cell line ROS 24/1 which lacks VDR (24), indicating that a nonclassic receptor is involved.

In summary, a substantial body of evidence now exists to indicate that at least some of the rapid 1α,25(OH)₂D₃-induced effects are transmitted by a membrane receptor, distinct from the classic VDR.

8. Triiodothyronine and thyroxine

Several mechanisms are described in the literature through which thyroid hormones may nongenomically induce cellular responses. However, receptors mediating such phenomena have not been definitely characterized. Some enzymes have been reported to be modulated in their activity by thyroid hormones, such as pyruvate kinase (12), where triiodothyronine (T₃) is more effective than thyroxine (T₄) in preventing tetramer formation and concomitant inhibition of enzyme activity. In another example, the less iodinated 3,5-diiodothyronine (3,5-T₂) binds to cytochrome-c oxidase, counteracting ATP inhibition of oxidase activity (10). T₃ is less effective, while T₄ and the noniodinated thyroxine have no effect at all. This mechanism may explain the short-term effect of thyroid hormones on mitochondrial respiration.

The activation of a crucial signaling enzyme, PKC, has been shown in vitro in erythrocytes (248). However, these experiments have not been performed with isolated PKC, so indirect effects are still possible.

High-affinity (Kᵦ) in the low nanomolar range or below) membrane binding sites for T₃ or T₄ have been described in mammalian liver (379), placenta (3), thyrocytes (429), and others as well as in avian embryonic synaptosomes (178). The latter system exhibits several properties characteristic for G protein-coupled receptors (GPCR), such as the reduction in T₃ binding capacity by GTPγS (177). In turn, T₄ and T₃ lower the GTPase activity, and upon ADP-ribosylation the thyroid hormones increased the formation of inactive Gₛ-GDP. The studies were done, however, on whole cell preparations, and the postulated GPCR was not identified.

Additional evidence for the possible involvement of a GPCR has come from the investigation of signal transduction pathways. T₄ activates the MAPK signaling line, but the effect is abolished by GTPγS or PTX (259). Here, only T₄, but not T₃, is active at physiological concentrations.

Partial identification of nonclassic thyroid receptor proteins has been reported only a few times. The affinity labeling reagent N-bromoacetyl-¹²⁵I-T₃ was found to react specifically with a 65-kDa protein, as judged from SDS-PAGE, in placenta (3). The protein, solubilized in its native state, is possibly a 140- to 150-kDa dimer as observed by gel filtration. On the other hand, the same reagent detected a 27-kDa protein in neuroblastoma plasma membranes (183).

A 43-kDa mass protein has been described in mitochondria that is related to the ligand binding domain of thyroid receptor (TR) α1 (529). This protein could bind to a thyroid responsive element and mitochondrial DNA sequences and was shown to bind TR with high affinity (86). No link has been made, however, to direct nongenomic action, which may be mediated through a 28-kDa protein (530).
In summary, although some hints on the identity of receptors for nongenomic thyroid effects have been acquired, complete identification or characterization is not yet achieved by far.

The possibility of classic receptors, or proteins closely related to, participating in the mediation of rapid effects has rarely been studied. However, many characteristics of responses or ligand binding observed are incompatible with classic receptors.

III. EFFECTS, SIGNALING PATHWAYS, AND CLINICAL IMPLICATIONS

A. Glucocorticoids

For about half a century, glucocorticoids play an important role in the treatment of autoimmune diseases, allergic diseases, and more recently in conditions that require the suppression of immune response, as for instance organ transplantation. Genomic effects of glucocorticoids have been extensively investigated during the past decades. They normally take place at low steroid concentrations in nanomolar ranges, as is the case under physiological conditions, and are mediated by cytosolic receptors that alter the expression of specific genes. Rapid glucocorticoid effects can be further divided into specific and nonspecific nongenomic effects (80). Both occur within seconds, but the latter ones only at high dosages of the glucocorticoid and are assumed to function via direct effects on cellular membranes and subsequent changes in ion transport. Specific nongenomic effects can be observed within seconds to minutes and seem to be mediated by steroid-selective membrane receptors (516). Rapid effects of glucocorticoids are investigated to a much lower extent than rapid effects of the other classes of steroid molecules, but they have been described at cellular levels, at the level of whole organ function, as well as at the level of whole physiological response.

Direct membrane effects of glucocorticoids have been shown already in 1974 in isolated hypothalamic synaptosomes (125). They were considered as the cellular counterpart for the negative feedback mechanism between plasma cortisol and ACTH. In hippocampal slice preparations of CA1 pyramidal cells, corticosterone influences the excitability as shown by increased spike amplitudes (398). In vitro experiments using guinea pig ganglion neurons demonstrated that glucocorticoid can hyperpolarize the membrane potential within 2 min (215). Incubation of rat cerebral cortex synaptosomes and of the human neuroblastoma line SK-N-SH with different glucocorticoids showed a rapid and dose-dependent increase of glutamate uptake (544). Because the effect could be blocked by an inhibitor of G protein activation, the authors conclude that G proteins might be involved in glucocorticoid activation in these cells. In Ishikawa human endometrial cells, Koukouritaki et al. (237) demonstrated rapid alterations in actin polymerization dynamics after treating cells for 15 min with 0.1 μM dexamethasone. In parallel, they observed a decreased cAMP content in treated cells and concluded a regulation of the processes by cAMP-dependent pathways. Because the glucocorticoid antagonist RU 486 completely abolished the effect, it seems to be mediated via specific glucocorticoid binding sites. However, transcriptional mechanisms were unlikely to be involved, since actinomycin D did not block the dexamethasone-induced actin polymerization.

Acute actions of dexamethasone on nicotine-induced intracellular Ca2+ transients, as well as nicotine-induced catecholamine secretion, were examined in porcine adrenal chromaffin cells (503). In the presence of 1 μM dexamethasone, both the nicotine-induced Ca2+ transient and catecholamine secretion were reduced by about 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 Ca2+ signaling (162). Similarly, corticosterone was found to inhibit the nicotine-induced Ca2+ influx with a half-maximal inhibitor concentration (IC50) of 0.61 μM 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 (388). In a similar manner, corticosterone inhibited the change in [Ca2+]i induced by high extracellular K+ in PC12 cells, but with the maximal effect seen at a relatively high concentration of 10 μM of the steroid (265). 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. Recently, again in PC12 cells, rapid nongenomic effects of corticosterone on neuronal nicotinic acetylcholine receptor have been shown (447). With the use of whole cell clamp technique the influence of corticosterone on the acetylcholine-induced current (IAcCh) was studied. Preincubation of these cells with corticosterone for 4 min had an inhibitory effect on the peak of the acetylcholine-induced inward current. The effect was reversible, concentration dependent, and voltage independent. Intracellular application of corticosterone did not affect the IAcCh. Extracellular application of corticosterone to the preincubated cells inhibited the curve amplitudes by ~49%. Corticosterone conjugated to BSA showed an inhibition similar to that caused by unconjugated corticosterone. Neither actinomycin D nor cycloheximide influenced the inhibition of acetylcholine-induced current by corticoste-
rone, thus suggesting a nongenomic mechanism mediating the above effects. The authors propose the binding of corticosterone to a specific site on the outer cell membrane, probably on the neuronal nicotinic receptor-coupled channel. The resulting inhibition of $I_{ACh}$ is supposed to control the immediate catecholamine release from sympathetic cells.

Rapid nongenomic effects that occur with high doses of dexamethasone, stabilizing membranes, could be shown in rat liver lysosomal membranes (212). Marker for lysosomal membrane integrity was the release of $\beta$-glucuronidase. Ten minutes after intravenous administration of 3 or 10 mg/kg dexamethasone, $\beta$-glucuronidase release was inhibited by 38 and 33%, respectively. A comparable inhibition was observed after 24 h. Pretreatment of rats with the glucocorticoid receptor antagonist RU 486 did not antagonize the short-term effect but almost completely prevented the 24-h membrane protection. The authors suggest a dual mechanism of high-dose dexamethasone on membrane integrity by rapid nongenomic effects and long-term receptor-dependent genomic events.

The nervous system is a field intensively investigated in regard to rapid glucocorticoid action. Rapid effects have been shown with regard to excitability, neuroendocrine responses, and behavioral tasks. Systemic glucocorticoid administration yielded rapid effects on neural activity in the hypothalamus of rat, rabbit, and cat (13, 149). In the limbic system of lizard serotonergic responses to systemically administered corticosterone were detected within 20 min after treatment, suggesting a possible mechanism for mediation of changing social behavioral events (408). Rapid corticosterone effects on neurophysiological processes and behavioral changes have also been extensively investigated in the amphibian Taricha granulosa. Multiple neurophysiological effects have been observed within 3 min of corticosterone injection, including a decline or cessation of firing of sensory responsive neurons and reduced excitability of reticulospinal neurons (403, 404).

In freely moving rats, either adrenalectomized or sham-operated, an intraperitoneal injection of 2.5 mg/kg corticosterone induced a transient increase in extracellular aspartate and glutamate levels in the CA1 area of the hippocampus within 15 min. The same fast and reversible effects were achieved by intrahippocampal corticosterone administration. Classic antagonists of intracellular glucocorticoid receptors or inhibitors of protein synthesis did not abolish the effect. Similar results were obtained with dexamethasone, whereas nonglucocorticoid steroids did not affect amino acid transmission in this hippocampal area (500). 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 $\mu$M rapidly (4 and 15 min, respectively) stimulated the Na$^+$-dependent uptake of glutamate (544). Corticosterone was also found to rapidly inhibit arginine vasopressin release in hypothalamic slices of rats at the smallest dose of 100 nM (262).

It has been suggested that rapid glucocorticoid actions may be involved in these phenomena because arginine vasopressin plays an important role in the regulation of hypothymalo-pituitary-adrenocortical axis activity. 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 486 (402). Corticosterone was found to rapidly affect $^{45}$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 $\mu$M (475).

Corticosteroids are assumed to influence behavioral changes associated with stressful events. Environmental perturbations can cause behavioral responses within minutes, and there is strong evidence that corticosteroids may induce changes by acting through nongenomic mechanisms. Taricha granulosa is known for rapid behavioral responses to stress, in a way that stress can suppress the courtship behaviors of Taricha (a prolonged amplexic clasp to facilitate receptivity in the female). This rapid effect is dependent on corticosterone and can be mimicked by corticosterone injection (311). Intraperitoneal injection of male Taricha with 32 nmol corticosterone suppressed sexual behavior within 8 min (352). In white-crowned sparrows, noninvasive corticosterone administration caused an increase in perch hopping within 15 min, indicating elevated locomotor activity that is consistent with behavioral responses to natural perturbations (70). In further experiments, it could also be demonstrated that the rapid behavioral responses to corticosterone varied with photoperiod and dose (72). Sparrows exposed to a long-day photoperiod showed increased activity in perch hopping at intermediate levels of corticosterone ($\sim$24 ng/ml), whereas in short-day exposed sparrows, the same dose level had no behavioral effect. The authors propose a less sensitive neural mechanism that justifies the behavioral response to corticosterone during a winter (short-day) photoperiod. Although intermediate levels of 24 and 40 ng/ml corticosterone increased the activity to threefold background values in the sparrows exposed to the long-day photoperiod, high physiological levels of corticosterone (65 and 97 ng/ml) did not induce any behavioral change. A model for nongenomic actions of glucocorticoids on behavior is shown in Figure 6.

The locomotor responses of rats in a novel environment could be increased by systemic corticosterone in-
have been shown to be activities of opioid receptor agonists. All of these effects produced by substantial stress (464). NO seems to be involved in this neurochemical mechanisms, since NO synthase inhibitors prevent these rapid effects. Inhibition of protein synthesis and blocking of intracellular receptors by specific receptor antagonists did not inhibit the rapid effects (419).

Apart from genomic glucocorticoid effects, membrane receptor-mediated mechanisms of glucocorticoids are assumed to participate in cellular processes leading to apoptosis. These mechanisms seem to involve damage of mitochondrial function by disruption of the mitochondrial membrane potential and decreased ATP availability (79). Functionally, there is evidence that at least in mouse S49 lymphoma cells, the human CCRF-CEM cell line and in lymphocytes of leukemic patients, membrane glucocorticoid receptors are necessary to initiate glucocorticoid-induced therapeutic apoptosis (117, 164, 168, 511). Membrane glucocorticoid receptors are present in normal and in cancerous lymphoid cells, and their actions may have important consequences for function and diseases of the immune system.

Further reports show a role of nongenomic glucocorticoid action in the inhibition of phosphate uptake in primary rabbit proximal tubule cells in a PLC/PKC-dependent way (357).

**B. Progesterone**

Rapid effects of progesterone have already been described in 1942 when Hans Selye found that an intraperitoneal application of progesterone induces a prompt onset of anesthesia in rats (437). Since then, many reports on nongenomic progesterone action followed, mostly in germ cells like sperm or oocytes.

*Xenopus* oocytes naturally arrest at the G2 phase of meiosis I. In response to progesterone, these cells are induced to enter the M phase. This process, known as meiotic maturation, is not affected by actinomycin D and occurred even in enucleated oocytes (284), suggesting a nongenomic effect. Only the early maturation events are discussed here, subsequent effects including a MAPK cascade and a Cdc2 activation are reviewed elsewhere (151). The initial biochemical responses of progesterone involve an inhibition of an oocyte adenyl cyclase and reduction of intracellular cAMP (152, 416). This decrease is thought to translate into a decrease in the activity of PKA (277). In addition, the protein kinase Eg2 becomes phosphorylated very early in the maturation process (7). These events of progesterone-induced maturation seem not to involve the α-subunits of classical G proteins, but rather the Gβγ subunits as the principal mediators (269, 446). In addition to *Xenopus*, progesterone also triggers the oocyte maturation in *Rana pipiens* oocytes involving a transient release of Ca\(^{2+}\) followed by a decrease in intracellular cAMP (236) and a transient rise in cGMP (235; for review, see Ref. 319).

Moreover, nongenomic progesterone effects have been intensively studied in human sperm but also in spermatooza from other mammals, e.g., mice (210), hamster (263), dog (453), and stallion (96). In addition to zona pellucida glycoprotein (ZP3), it has been demonstrated that progesterone is a natural stimulus of the acrosome reaction in sperm (292, 353). This event is mediated by a rapid increase of intracellular [Ca\(^{2+}\)]\(_i\) occurring within seconds after addition of the steroid (15, 293). The maximal increase was detected at relatively high progesterone concentrations of 1–10 μM (58) which, however, naturally occur in the cumulus matrix surrounding the oocyte (17). The signal transduction mechanisms that are involved in progesterone-induced effects on [Ca\(^{2+}\)]\(_i\) and the acrosome reaction have been reviewed in detail elsewhere (360, 384). Interestingly, the potent antagonist of the intracellular PR, RU 486 (10 μM), has been found to decrease [Ca\(^{2+}\)]\(_i\) when given alone thus partially antagonizes the effect of progesterone on [Ca\(^{2+}\)]\(_i\) and sperm acrosome reaction (535). However, due to the similar inhibition of the thapsigargin-induced Ca\(^{2+}\) elevation by RU 486, it has been speculated that the antagonist does not necessarily compete with progesterone for the same binding sites to exert its inhibitory action (439).
was not affected by RU 486, but the concentration of 1 μM (15) perhaps was too low.

Moreover, progesterone also promotes Na⁺ (155, 361) and Cl⁻ fluxes (490) in spermatozoa which may be linked to the depolarizing effect of progesterone (300).

The finding that progesterone induces the human sperm acrosome reaction has pushed speculations on the clinical significance of this effect. In this regard it has been shown that sperm responsiveness to progesterone is reduced in oligozoospermic and infertile patients (16). Moreover, progesterone binding to the acrosomal membrane has been suggested to be a valuable predictor for outcome of in vitro fertilization. High progesterone binding to sperm was linked to a higher success rate of fertilization (161).

In addition to germ cells, a number of nongenomic progesterone effects have also been described in several other tissues or cells. For example, in isolated neocortical slices, progesterone (0.1 μM) augmented forskolin-induced cAMP production within 15 min without prior treatment with estrogen. In these studies progesterone alone was ineffective, the steroid also failed to affect norepinephrine- or isoproterenol-induced cAMP increase (2). In contrast, preincubation of rat hypothalamic slices with progesterone (20 nM) for 5 min significantly suppressed norepinephrine-stimulated cAMP formation by >60%. This effect was estrogen dependent in that progesterone in vitro did not inhibit NE-stimulated cAMP accumulation in slices from ovariectomized rats not pretreated with estradiol (371). In addition, progesterone was found to decrease cAMP levels in quiescent adipocytes or in adipocytes stimulated by isoproterenol (470).

Rapid Ca²⁺ fluxes in response to progesterone (10 pM to 10 nM) which are modulated by PLC-β1 and PLC-β3 and involve the Goα11 subunit have been demonstrated in female and male osteoblasts. 17α-Hydroxyprogesterone was as active as progesterone and medroxyprogesterone had no effect. In addition, a rapid progesterone-induced increase in the formation of inositol 1,4,5-trisphosphate (IP₃) and 1,2-diacylglycerol (DAG) was observed in these cells (191, 251). Similarly, progesterone triggers an immediate and transient peak in [Ca²⁺], and an induction of IP₃ in porcine granulosa cells (257, 272). The mentioned effects in osteoblasts and granulosa cells have been shown to be insensitive to RU 486 and were also triggered by progesterone bound to BSA, although at 100-fold higher concentrations. This always opens the possibility that a 1% impurity of free steroid is responsible for the rapid responses, rather than the impermeable compound (see also sect. v).

Recently, progesterone (0.5 μM) was found to blunt KCl-induced elevation of [Ca²⁺], and to decrease L-type calcium channel current in vascular smooth muscle cells (VSMC) (28). The steroid (1 μM) has also been described to rapidly increase and induce membrane depolarizations and inward currents in thymic epithelial cells (209). In addition, similar concentrations of the steroid cause an immediate rise in [Ca²⁺], in a murine Leydig tumor cell line (mLTC-1) which seems to be devoid of PR (129).

Several other reports deal with rapid progesterone effects on ion fluxes. However, their physiological significance is uncertain since unphysiologically high steroid concentrations were used in the studies. For example, it has been described that progesterone (50 μM) decreases Ca²⁺ currents in a human intestinal smooth muscle cell line (54). Moreover, micromolar concentrations of progesterone rapidly block voltage-gated and Ca²⁺-activated K⁺ channels, resulting in depolarization of the membrane and inhibition of Ca²⁺ signaling in T lymphocytes (127). This provides a nongenomic mechanism for progesterone-mediated immunosuppression in the placenta. For the sake of completeness, it should be mentioned that rapid electrophysiological effects of progesterone have been described in Leydig cells (406), rat hepatocytes (504), natural killer cells (278), and CA1 hippocampal neurons (222).

Progesterone has been demonstrated to modulate vasorelaxation after acute application. For example, progesterone at micromolar concentrations induces a dose-dependent relaxation of rat uteri (precontracted with KCl) (81, 197) and saphenous artery segments (precontracted with norepinephrine) within 10 min (225) and rapidly decreases the contractile activity of murine jejunum (347). However, acute treatment with progesterone at physiological concentrations reduces bradykinin- and calcium ionophore A23187-mediated relaxations in porcine coronary artery rings (477).

Progesterone is considered to be involved in maintaining pregnancy by depressing the uterotonic action of the peptide hormone oxytocin. In this context, it was postulated that progesterone binds to the rat oxytocin receptor (OTR), a member of the GPCR family, with high affinity (Kᵦ ~20 nM). Moreover, application of progesterone (10 nM-1 μM) to cells expressing this receptor caused an inhibition of the oxytocin-induced Ca²⁺ response (187). Interestingly, in CHO cells expressing recombinant human OTR, no inhibition of oxytocin binding by progesterone (up to 10 μM) was observed. Instead, a progesterone metabolite, 5β-pregnane-3,20-dione, inhibited oxytocin binding with an inhibition constant of 32 nM (187). In contrast, Burger et al. (77) found that within minutes high concentrations of progesterone (>10 μM) attenuated or blocked the signaling of several GPCRs, including the OTR.

Nongenomic progesterone effects have also been described in conjunction with sexual behavior (115). For example, activation of sexual receptivity in rats occurs within 10 min of intravenous infusion of progesterone (20–400 μg) (303). In ovariectomized, estradiol-primed mice, an intravenous progesterone (200 μg) infusion sig-
significantly increased lordosis within 10 min. Interestingly, this effect was seen in both PR KO and wild-type mice (160). Recently, it has been shown that the actions of progesterone in the ventromedial hypothalamus require intracellular PR but, interestingly, in the ventral terminal they do not. In the ventral tegmental, progesterone may facilitate lordosis after metabolism to and/or biosynthesis of 3α,5α-THP, which may have subsequent actions at other receptors like GABA/ benzodiazepine receptor complexes (159).

Recently, it has been described that 17β-estradiol- and progesterone-BSA bind to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). In this context, progesterone (100 nM) was found to rapidly decrease V_max of the enzyme, whereas Michaelis constant (K_m) was not changed (221).

Like other steroids, progestins also activate MAPK. In T47D cells, which are rich in PR_A and PR_B and also contain ER, treatment with R 5020 (10 nM) resulted in a rapid stimulation of ERK-2 activity, which was already detectable after 2 min. The effect was inhibited by the use of the antiprogestin RU 486 (1 mM) and, surprisingly, by various anti-estrogens. In T47D Y cells, which lack PR but are endowed with high ER levels, no stimulation of ERK-2 activity by R 5020 was found, suggesting that the PR is required for this effect (305). The rapid activation of the c-Src/p21ras/MAPK signaling pathway has been described to depend on the ability of the intracellular PR to interact with SH3 domains (61).

C. Estrogens

One of the first observations on nongenomic actions of estrogens has been produced in the female reproductive system. The endometrium is a major target for ovarian steroids, and steroids play an essential role in regulating growth and differentiation in the endometrium leading to dynamic morphological and functional changes. In cultured endometrial cells, 17β-estradiol rapidly (<10 min) stimulates Ca^{2+} influx (375), which was confirmed recently (366). Accordingly, putative membrane estrogen receptors on the cell surface could be detected in endometrial cells (376). 17β-Estradiol also elicits functional changes in the endometrial cells by increasing the number and density of microvilli. These results could be supplemented by findings that 17β-estradiol-induced structural changes are biphasic: they appear to be secondarily enhanced after 7 min (391). Rapid increases in [Ca^{2+}], could also be found in maturing human oocytes and granulosa cells after addition of only 0.1 nM 17β-estradiol (318, 479). These effects could be induced by 17β-estradiol, estrone, 17α-estradiol, and estriol, whereas progesterone, pregnenolone, testosterone, androstenedione, and 5α-dihydrotestosterone were ineffective. Moreover, the estrogen-induced [Ca^{2+}] increase was not affected by the ER agonist/antagonist tamoxifen or by the RNA and protein synthesis blockers actinomycin D or cycloheximide (400), which is in contrast to findings in other tissues. The estrogen-induced [Ca^{2+}] increase in granulosa cells is inhibited by pretreatment with inhibitors of the inositol phospholipid hydrolysis like neomycin and U 73122 (444). Further investigations revealed that 17β-estradiol may directly influence the quality of maturing oocytes. The addition of 17β-estradiol did not influence the progression of oocytes through meiotic maturational, but it improved the fertilization potential of the in vitro matured oocytes (479).

As already mentioned in section II.3, estrogen binding sites are also present on human sperm. Because spermatozoa are almost devoid of protein de novo synthesis, and sperm protein synthesis is confined to the mitochondria, effects mediated by estrogens can be linked to non-genomic actions. Previous studies have shown that 17β-estradiol induces functional changes in sperm by the enhancement of sperm motility (39, 95). Moreover, in spermatozoa of fertile and infertile men, the addition of 17β-estradiol is claimed to improve the results of the zona-free hamster ova penetration test (91). A recently published study revealed that 17β-estradiol alters the testicular androgen production by causing a decrease in gonadotropin-stimulated 11-ketotestosterone synthesis in testes of Microgogonas undulatus (264).

The detection of estrogen receptors in the brain and estrogen-concentrating cells in the pituitary gland, hypothalamus, and other brain regions (372) has shifted the interest to estrogen effects, which are not directly connected to reproduction, and there is increasing evidence that estrogen actions in the brain are mediated in part by non-genomic mechanisms. Within seconds after application, 17β-estradiol led to a brief hyperpolarization and increased potassium conductance in rat medial amygdala neurons, an effect which persisted after suppression of protein synthesis. In addition, a greater proportion of the neurons from females than males were affected by 17β-estradiol (325, 537). The rapid time course and the insensitivity to protein synthesis inhibitors led to the assumption that genomic effects may not be involved. Moreover, it is now well established that estrogens act via interaction with specific receptors at the plasma membranes of neurons (69, 484 and others), followed by the activation of intracellular signal transduction pathways. Further investigations have shown that estrogens stimulate the formation of cAMP (194), the phosphorylation of CREB (543), and the formation of IP_3 (148) and activate the MAPK signaling pathway in a neuroblastoma cell line (513).

Previous studies have revealed that estrogens may have an important role in the differentiation of mouse midbrain dopaminergic neurons via nongenomic mechanisms, which includes the interaction with a membrane
receptor coupled to intracellular signaling pathways. In previous studies, the addition of 17β-estradiol evoked a selective increase in newly synthesized dopamine (359) and dopamine release (40). In cultured embryonic dopaminergic neurons, 17β-estradiol evoked a rapid Ca\(^{2+}\) release from intracellular stores in cultured embryonic dopaminergic neurons reaching a peak rise within 5 s. The BSA-estrogen conjugate was equally effective in triggering the typical rise in [Ca\(^{2+}\)]\(_i\) (53). Moreover, estrogens modified within seconds after application basal firing rates (97) and led to rotational and stereotypic behavior in adult rodents (40). It could be further demonstrated that 17β-estradiol and the BSA-estrogen conjugate enhanced neurite growth and arborization of dopaminergic neurons. This effect was inhibited by antagonists of cAMP/PKA and Ca\(^{2+}\) signaling pathways, but not by the ER antagonist ICI 182780 (52). With the use of quantitative double-labeling immunocytochemistry and gel shift assay, it could be demonstrated that 17β-estradiol exposure induced the phosphorylation of CREB in midbrain dopaminergic cells. Again, this effect was antagonized by the simultaneous application of inhibitors of the cAMP/PKA or Ca\(^{2+}\) pathways, but not the ER antagonist ICI 182780 (52).

Several lines of evidence indicate that estrogens possess neuroprotective properties, which may be induced by genomic as well as nongenomic mechanisms. Physiological concentrations of 17β-estradiol rapidly intensified the release of secreted amyloid β-precursor protein (APP) in mouse hippocampal HT22 and human neuroblastoma SK-N-MC cells. The synthesis of amyloid β and its precursor APP is a central step in the pathogenesis of Alzheimer’s disease. Again, the rapid secretion of APP is mediated through 17β-estradiol via the phosphorylation of ERK1/2, which are both members of the MAPK pathway (279), indicating a cross-talk between genomic and nongenomic actions. Interestingly, the activation MAPK signaling pathway and the increased release of APP induced by 17β-estradiol was independent of the ERs, as it occurred also in neuronal cells lacking a functional ER (279). In a recent study it could be shown that estrogen has an antiapoptotic effect on primary cortical neurons that was mediated through the PI 3-kinase/Akt signal transduction pathways. The PI 3-kinase/Akt signals evoked the phosphorylation of CREB at a serine residue and the upregulation of the antiapoptotic protein Bcl-2. The neuroprotection of estradiol could be inhibited by the upregulation of the antiapoptotic protein Bcl-2. In a recent study it could be shown that estrogen has an antiapoptotic effect on primary cortical neurons that was mediated through the PI 3-kinase/Akt signal transduction pathways. The PI 3-kinase/Akt signals evoked the phosphorylation of CREB at a serine residue and the upregulation of the antiapoptotic protein Bcl-2. The phosphorylation of CREB in midbrain dopaminergic cells. Again, this effect was antagonized by the simultaneous application of inhibitors of the cAMP/PKA or Ca\(^{2+}\) pathways, but not the ER antagonist ICI 182780 (52).

Rapid and specific effects of 17β-estradiol have been also demonstrated in isolated duodenal cells, initiated on the cell surface and resulting in the activation of the PI 3-kinase/Akt pathway and Ca\(^{2+}\) influx through dihydropyridine-sensitive Ca\(^{2+}\) channels (373). Furthermore, the PLC inhibitor neomycin and U 73122 suppressed the stimulatory effect of 17β-estradiol on the Ca\(^{2+}\) influx into duodenal cells, indicating an involvement of the PLC (374). Recently, evidence could be provided that 17β-estradiol indirectly stimulates the cytosolic PKA activity in female rat distal colonic epithelium. Basal PKA activity was stimulated by cAMP, which was abolished by the addition of the specific inhibitor PKI and the adenylyl cyclase inhibitor SQ-22536. In contrast, the PKC was directly stimulated by 17β-estradiol; the specific PKC inhibitor GF 109203X prevented the 17β-estradiol-induced PKA activation. The PKC and PKA inhibitors abolished the increase in [Ca\(^{2+}\)]\(_i\). These effects were only demonstrated in female rats; 17β-estradiol failed to induce a similar effect in membrane or cytosolic fractions of male rats (121). Gender-related differences could be also observed in the regulation of Cl\(^-\) secretion in the rat distal colonic epithelium. 17β-Estradiol inhibited colonic Cl\(^-\) secretion, which involved intracellular Ca\(^{2+}\) and PKC. The ability of
17β-estradiol to reduce the Cl− secretion in the female colon may probably contribute to the clinically important 17β-estradiol-induced salt and water retention in females (105).

Clinical and experimental data suggest that estrogens may play a role in the genesis and perpetuation of colorectal cancer (130, 367). The mechanism by which estrogen exerts proliferative properties has been assumed to be exclusively mediated by genomic actions. Interestingly, 17β-estradiol induced a rapid cellular alkalization of crypts and cancer cells that was sensitive to Na+/H+ exchanger (NHE) blockade or PKC inhibition. Thymidine incorporation drastically increased both in crypts and cancer cells, which, again, could be inhibited by NHE blockade, indicating a nongenomically mediated mitogenic effect of 17β-estradiol (527).

In a previous investigation, 17β-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 (74). In male and ovariectomized mice, 17β-estradiol seems to be able to prevent the development of diabetes mellitus (126), an effect which could be probably mediated by a nongenomic mechanism. At physiological concentrations, 17β-estradiol rapidly and reversibly closes KATP channels from pancreatic β-cells and depolarizes the plasma membrane resulting in increased [Ca2+]i, which in turn enhances the insulin secretion (327).

In human and pig coronary arteries and rat aortas, 17β-estradiol and 17α-estradiol induce an acute and clinically relevant relaxation (399,418). Furthermore, 17β-estradiol leads to an acute relaxation of rat arteries from females, but not males (291). Within minutes, 17β-estradiol increases basal intracellular cAMP in pulmonary vascular smooth muscle cells, which is probably caused by an estrogen-induced activation of membrane adenylate cyclase (146). In PC12 cell membranes, 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 (94). Furthermore, in PC12 cells, 17β-estradiol rapidly reduces the catecholamine secretion by inhibiting L- and N-type Ca2+ channels and P2X2 receptors (232).

As demonstrated in BAEC, 17β-estradiol induces a translocation of eNOS from the plasma membrane to intracellular sites close to the nucleus. This effect of 17β-estradiol was detected at concentrations as low as 1 pM. Moreover, cGMP release could be rapidly increased by 17β-estradiol, and this effect could be blocked by the ER antagonist ICI 164384 (182). Also in BAEC, 17β-estradiol (5 nM) increased NO production through ERα localized in specific plasma membrane domain caveolae. The 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 17β-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 17β-estradiol. The BSA-17β-estradiol conjugate also increased the NO concentration, and the ERα antagonist ICI 182780 completely blocked estrogen-stimulated NO release, which supports the assumption that a plasma membrane receptor similar to ERα is involved in these effects (231). A model for the mode of nongenomic modulation of NO synthesis in caveolae is given in Figure 7.

Further effects of estrogens on vascular tissues have been already described in section μD3.

In addition to the estrogen effects described in his section, estrogen binding sites in plasma membranes could be found in various tissues like myometrium, liver, a breast cancer cell line (MCF-7), and on the cell surface of osteoblast and osteoclast-like cells (51, 153, 376).

As described in detail in section μB, progesterone facilitates the human sperm acrosome reaction, an effect which could broaden the therapeutic options for male infertility. The discovery of functional ERs in the surface of human sperm and the inhibition of progesterone-induced acrosome reaction by estrogens might be pathophysiological relevant for the appropriate timing of capacitation and the acrosome reaction. A future therapeutic concept for male infertility could be the inhibition of the sperm ER while enhancing the progesterone effect.

For many years, hormone replacement therapy appeared to be a potential cardioprotective agent in coronary heart disease, the leading cause of death in developed countries. Beneficial therapeutic effects could be derived from the 17β-estradiol and progesterone actions to immediately relax small and large arteries. However, recent clinical trials have not elicited any advantageous effects of hormone replacement therapy on risk for major cardiovascular events among women with established coronary heart disease.

The investigations of steroids in the nervous system have emphasized the fact that estrogens possess neuroprotective, antiapoptotic, and antioxidant effects that may be in part induced by nongenomic mechanisms. The study of estrogen actions in the brain might be a challenging topic and a potential target for pharmaceutical development.

D. Androgens

Rapid effects of androgens on [Ca2+]i have been intensively studied in several systems. In male rat osteoblasts, testosterone (10 pM to 10 nM) increased [Ca2+]i within 5 s via Ca2+ influx as well as via Ca2+ mobilization from the endoplasmic reticulum. In addition, IP3 and DAG formation was induced within 10 s after addition of sim-
ilar concentrations of the steroid (255). Interestingly, comparable amounts of testosterone did not elicit an increase in \([\text{Ca}^2+]/\text{H}11001\) in female rat osteoblasts (256). In primary cultures of neonatal rat myotubes, testosterone (50–100 nM) induced an increase of \([\text{Ca}^2+]/\text{H}11001\) within seconds. This response was frequently accompanied by irregular oscillations. In this system, testosterone also produced a rapid increase in IP3 levels that reached a peak threefold higher than basal level (133). Moreover, physiological concentrations (1–10 nM) of testosterone were described to rapidly raise \([\text{Ca}^2+]/\text{H}11001\) in activated T cells. Contrary to the effects in osteoblasts, the testosterone-induced increase of \([\text{Ca}^2+]/\text{H}11001\) in these cells is solely resulting from \(\text{Ca}^{2+}\) influx (44, 45). In this context it is worthwhile to note that classic ARs in splenic T cells appear to be genomically unresponsive to testosterone. On the other hand, the postulated cell surface receptors for testosterone are described to be functionally active.

The finding that T cells respond to testosterone in a nongenomic manner suggests an as yet unexploited option for a modulation of T-cell activity with possible applications in proliferative and infectious diseases. For example, the binding of the androgen to surface receptors of T cells followed by an immediate \(\text{Ca}^{2+}\) influx may be the initial event in the testosterone-induced susceptibility to \(\text{Plasmodium chabaudi}\) malaria in mice (45). If this finding could also be demonstrated in humans for other plasmodium species, it may be speculated that selective inhibition of testosterone T-cell surface receptors may open a new path of preventing malaria infections.

The effects of testosterone on \([\text{Ca}^2+]/\text{H}11001\) described above are not inhibited by nuclear AR antagonists like hydroxyflutamide or cyproterone, suggesting an action independent of classic AR. This argumentation is further supported by findings in mouse IC-21 macrophages where the classic AR is absent. In these cells, testosterone likewise induced a \([\text{Ca}^2+]/\text{H}11001\) increase that was found to be predominately due to release of \(\text{Ca}^{2+}\) from intracellular stores (46).

Moreover, studies with regard to androgen-induced \(\text{Ca}^{2+}\) fluxes have been done in immature rat Sertoli cells, which contain the normal AR, in the human prostatic...
tumor cell line (LNCaP), which contains a mutated AR, and the human prostatic cell line PC3, which does not contain AR (270). Testosterone as well as the synthetic androgen R 1881 (both 1–1,000 pM) rapidly induced transient Ca$^{2+}$ increases in all cell types. These responses could be inhibited by hydroxyflutamide but, in contrast to effects on the nuclear receptor, were not inhibited by cyproterone acetate.

In addition, testosterone, although at nonphysiological high concentrations (0.3–3 μM), increased [Ca$^{2+}$], in freshly isolated Sertoli cells in a hydroxyflutamide-sensitive manner (186). A similar effect has been shown by using other androgens like nibolerone (dimethylnortestosterone) and 5α-dihydrotestosterone in LNCaP cells (465).

Furthermore, androstenedione, the main intrafollicular androgen, rapidly increased [Ca$^{2+}$], in human granulosa luteinizing cells (273) and porcine ovarian granulosa cells (257). This increase was dose dependent, with maximum activity at 1 nM androstenedione in pig and 10 nM in human cells. In both cases, comparable concentrations of testosterone had no effect, and the nuclear androgen receptor antagonist flutamide was ineffective. In human granulosa cells, the increase in [Ca$^{2+}$], resulted from both Ca$^{2+}$ influx and mobilization from endoplasmic reticulum, whereas in porcine cells, this effect is solely dependent on intracellular stores. In both cases, G protein-mediated PLC activation appears to be involved.

Similar to estrogens and progestins, androgens were found to stimulate MAPK (also called ERK) in several cells. The androgen R 1881 stimulates activity of ERK promptly after addition of the steroid (1 nM) in human PMC42 breast cancer cells (545). Unlike anti-estrogens and anti-progestins, which inhibit agonist-induced ERK activation (305), the anti-androgen flutamide stimulates ERK activity in the same manner as R 1881. In this context, dihydrotestosterone (100 nM) was also shown to rapidly induce phosphorylation of ERK1 and ERK2 in primary prostatic stromal cells and LLNCaP cells but not in human genital skin fibroblasts, another androgen-responsive cell (370). This dihydrotestosterone-induced stimulation of ERK was also found in human prostate PC3 cells, which were stable transfected with wild-type AR, but not in mock-transfected cells, suggesting an involvement of the classic AR. Like in PMC42 cells, the antiandrogens hydroxyflutamide and casodex (100 nM and 10 μM) alone were able to induce ERK phosphorylation (370). Therefore, the antiandrogens currently available as therapeutics may not totally abrogate all androgenic activity in target cells. These observations may help to explain the clinical phenomenon of withdrawal responses observed in patients suffering from prostate cancer who become refractory to these antagonists (294, 422).

Androgen effects have also been described in cardiac tissue. In the isolated and perfused rat heart, testosterone (0.1–100 nM) blocked the adenosine vasodilator effect and increases vascular resistance (89). Although the studies were done in a time range of 1 h, actinomycin D and cycloheximide were ineffective in blocking these effects.

On the contrary, similar concentrations of testosterone were found to induce a vasodilatation in canine coronary conductance and resistance arteries in vivo (98). With the use of supraphysiological concentrations of testosterone (25–300 μM), direct vasorelaxing effects on rat thoracic aorta have been described (107). Similarly, the androgen 5β-dihydrotestosterone has been found to produce relaxation of rat aorta rings precontracted by nor-epinephrine or K$^+$ (368). However, because of the high concentrations used, the specificity is doubtful.

Several other rapid androgen responses have been described. For example, the effects on the electrical activity of neurons have already been reviewed elsewhere (144, 516). More recently, the testosterone-induced modulation of Cl$^-$ secretion in cultured rat efferent duct epithelia was analyzed by the use of the short-circuit current ($I_{sc}$) technique (252). Testosterone rapidly reduced forskolin-stimulated $I_{sc}$ with an IC$_{50}$ value of 1 μM. Cyproterone acetate or flutamide did not block the effect. Furthermore, micromolar concentrations of testosterone reduced the forskolin-induced rise of intracellular cAMP in these cells (252). In addition, it has been demonstrated that physiological concentrations of testosterone or testosterone-BSA (0.1 nM to 1 μM) can elicit significant prolactin release from lactotrophs (type 2) in the male rat pituitary within 5 min (101). Testosterone (100 nM), like progesterone and 17β-estradiol, was also found to depress gonadotropin releasing hormone receptor mediated stimulation of low-K$_m$ GTPase activity in anterior pituitary membranes from male rats (393) and to increase glycogen phosphorylase activity in the liver (119).

E. Neurosteroids

Neuroactive steroids have been suggested to influence physiology and pathophysiology of the central nervous system in various fields. They control regulation of gene expression after binding to intracellular receptors, which may act as transcription factors. Neuroactive steroids, however, have also been shown to be potent modulators of an array of ligand-gated ion channels and specific G protein-coupled receptors nongenomically. As the foundation for their effects, an intracellular cross-talk between nongenomic and genomic actions of neuroactive steroids has been suggested. The possible physiological and pathophysiological role of neuroactive steroids has been proposed and explored in circumstances such as sleep, memory function, neuroprotection, depression and anxiety disorders, reaction to stress, premenstrual syndrome, and addiction diseases. These aspects will be briefly reviewed in the following paragraphs.
In sleep disorders, clinical experience has been gathered in studies investigating GABA\textsubscript{A} receptor modulating neuroactive steroids. Progesterone has been proposed to shorten sleep latency and to increase non-rapid-eye-movement sleep (non-REM) as well as sleep duration (157). The hypothesis that allosteric modulation of the GABA\textsubscript{A} receptor may play a significant role also in sleep disturbances in humans is backed by the finding that DHEA-S, an allosteric inhibitor of GABA\textsubscript{A} receptors, is markedly decreased in patients with chronic fatigue syndrome (241).

Investigators have also made progress in examining the sleep-modulating properties of DHEA. After oral administration of DHEA, an increase in REM sleep can be observed while simultaneously EEG activity in the sigma frequency range during REM sleep is enhanced. This finding which may seem puzzling at first sight is compatible with a mixed GABA-agonistic/antagonistic profile of DHEA as a modulator of the GABA\textsubscript{A} receptor (158). However, high doses of systemic DHEAS, the sulfate ester of DHEA, did not affect sleep structure in rats in another study, whereas a dose-dependent modulation of electroencephalogram (EEG) patterns was seen during non-REM sleep (423). DHEAS (50 mg/kg) significantly increased EEG power in the frequency range of sleep spindles, and 100 mg/kg depressed EEG power in the slow-wave frequency bands. It has been hypothesized that additionally to GABA\textsubscript{A} receptor modulation, glutamate-induced currents may be responsible for conferring this effect of DHEAS. The GABA agonistic properties of neurosteroids have led researchers to investigate a possible role of these compounds as therapeutic modulators of unfavorable sleep-wake rhythms. Compared with placebo, the group receiving 15 mg/kg alloptoprenanolone over a treatment period of 5 days exhibited shorter non-REM sleep latencies, prolonged REM sleep latencies, and longer non-REM sleep episodes in rats (109). This finding is comparable to the action of other agonistic modulators of the GABA\textsubscript{A} receptor such as short-acting benzodiazepines. However, in this study, no development of tolerance with respect to sleep-modulating characteristics was observed, which would have been expected for other known drugs targeting the benzodiazepine binding site of the GABA\textsubscript{A} receptor.

One of the major problems in this field of research is the lack of information how neuroactive steroids may modulate sleep-wake behavior and EEG patterns in humans. This is particularly troublesome since the steroids that have been shown to modulate these aspects in the animal model have hardly been investigated at all in humans. The limited evidence gathered so far derives primarily from rather small studies in postmenopausal women with and without hormone replacement therapy. Sleep latency, distribution of sleep stages, sleep efficiency, and total sleep time were very similar in a study investigating transdermal estrogen replacement compared with placebo in 62 postmenopausal women (380). Women receiving estrogen, however, reported a subjectively better sleep quality than women on placebo, suggesting effects beyond a modulation of GABA or glutamergic receptors shown for other neuroactive steroids. This finding slightly contrasts the results of another small study investigating sleep patterns and corresponding EEG changes in 11 postmenopausal women. Transdermal estrogen enhanced REM sleep duration, reduced time awake during the first two sleep cycles, and restored normal sleep EEG patterns compared with women not receiving estrogen replacement therapy (8). The differences of these two studies may have originated in different baseline stress levels of women in the two studies. The procedure of taking the EEG may have elevated the baseline stress level with an associated increase in cortisol levels. This is suggested by a study of Prinz et al. (383), who found that elevated urinary free cortisol is associated with impaired sleep and earlier awakening in postmenopausal women without estrogen replacement therapy, but not in women on estrogen replacement therapy. These studies illustrate the current dilemma of evaluating the effects of neuroactive steroids in sleep studies in humans. Diverse study populations, and the lack of in vitro investigations for the steroids tested in vivo, have so far hardly been able to accurately test the proposed hypotheses. Instead, the complexity of the matter has only just become obvious with studies generating more questions than answers.

Memory involves a complex interaction of many different types of receptors and thus is modulated by several pathways. DHEA and its sulfate DHEAS have been described to enhance memory, an effect that can in part be explained by the modulation of sleep patterns via the GABA\textsubscript{A} receptor. Other pathways include interaction at the NMDA receptor, as suggested for pregnenolone. This compound as well as its sulfate and DHEA caused improvements in avoidance training and other test paradigms in rodents (285). As pregnenolone sulfate also reduces scopolamine-induced learning deficits and memory impairment caused by a selective NMDA antagonist, an allosteric action at this receptor is likely (286, 304). Sigma receptors also participate in memory, as the memory-enhancing effect of DHEAS and pregnenolone sulfate can be blocked by a sigma receptor antagonist (395). However, memory enhancing and neuroprotection (see below) cannot always be separated from each other, particularly in elder individuals, and many neuroactive steroids exhibit both effects. A different mechanism underlying steroid action in memory processes may be the modulation of excitatory synaptic transmission by estrogens, particularly of glutamate receptors in the hippocampus (195). In the context of neuroprotection, the administration of antagonists at the NMDA receptor, such as the syn-
thetic 5β-pregnan-3-ol-20-one 3-hemisuccinate, has been shown not only to inhibit NMDA-induced currents, but also to prevent cell death in primary cultures of rat neurons. The same steroid when administered intravenously reduces infarct size after focal cerebral ischemia (514). Protection against NMDA-induced neuronal death is also inferred by 17β-estradiol by direct inhibition of the NMDA receptor (515). The clinical importance of such phenomena is supported by epidemiological studies that revealed a decreased risk and severity of dementia in women on estrogen replacement therapy (321). Other studies have correlated decreased levels of neurosteroids to various forms of dementia (32). The NMDA receptor complex also is targeted by many nonselective sigma receptor ligands, although selective sigma ligands show neuroprotective action as well, presumably by preventing the release of excitotoxic amino acids (288). Other mechanisms of neuroprotection may involve the activation of MAPK by estrogens and several progestins (334). The relevance of this pathway is underlined by the abolishment of estrogen neuroprotection by MAPK inhibitors (452). The clinical use of steroids for memory enhancement and neuroprotection is currently under investigation (246).

Depression as a disorder of mood often is associated with anxiety. In this context, anxiolytic properties that have been demonstrated for several steroids are of great physiological importance. In rodents, the administration of positive allosteric GABA<sub>A</sub> modulators comprising allopregnanolone (56, 549), tetrahydrodeoxycorticosterone (THDOC; Ref. 108), alphaxolone (73), and ganaxolone (42) all showed such action under various conditions. Remarkably, anxiolysis seems to be independent from sedative properties associated with some of the steroids. Despite the numerous studies done in rodents, hardly any clinical trials of possible anxiolytic properties of this class of compounds have been reported.

During one of the few controlled, double-blind studies, progesterone was shown to improve many symptoms of women suffering from premenstrual syndrome (116), including alleviation of depression. Because progesterone is metabolized to many different neuroactive steroids, it is difficult to determine which hormone(s) ultimately confer the effect. Some evidence points to the GABA<sub>A</sub> modulator 5α-pregnanolone (495), but there is no clear correlation between steroid levels and clinical symptoms (426). However, the alleviation of symptoms of depression by tricyclic antidepressants has been attributed to the correction of a disequilibrium of neuroactive steroids (401).

Beneficial effects on depressive syndromes have also been shown for steroid agonists at the sigma-1 receptor (395). Apart from depressive disorders, anxiolysis is also beneficial in stress conditions. Consequently, allopregnanolone and THDOC elicit marked antistress effects in addition to their anxiolytic properties. Stress conditions are supposed to downregulate GABAergic neurotransmission and induce anxiety-like states (55). Changes in the levels of neuroactive steroids are supposed to occur in response to such events, indicating a close relation of stress, neurosteroid levels, and GABA receptor function (27). For example, elevated levels of corticosterone, which may be present during stress conditions, have been shown to affect the modulation of ligand binding to GABA<sub>A</sub> receptors by THDOC and GABA itself (349) and altering the hippocampal inhibitory tone.

There are numerous and sometimes contradictory reports on the etiology of premenstrual syndrome (PMS). While some studies established a correlation between a decline of the levels of the anxiolytic, GABA-modulating allopregnanolone and adverse symptoms such as anxiety, other studies failed to find such a relationship (457). Replacement therapy with allopregnanolone or its precursor progesterone were not reliably successful. Estradiol levels have been correlated with PMS symptoms as well, because this steroid has been associated with excitatory effects, possibly experienced as anxiety. Estradiol effects may occur, however, at increased steroid levels as well as on withdrawal and appear to be complex. Interestingly, the action of selective serotonin uptake inhibitors may be mediated at least in part by a direct increase in allopregnanolone synthesis from progesterone (190).

For addiction diseases, the data obtained on improvement of mood disorders and other psychosocial syndromes by steroid administration suggest their possible use for alleviating the problems associated with drug withdrawal. In a clinical study, however, progesterone was ineffective in improving symptoms of benzodiazepine withdrawal in patients having taken diazepam for more than 1 yr. In contrast, animal models of drug dependence identified allopregnanolone as effective in reducing anxiety and hyperlocomotion (396). Interesting results on the mechanism of clinical addiction development were obtained in rodent models. Gender differences were observed in the rapid augmentation of behavioral responses to stimulants such as amphetamine and cocaine (41). While estrogen enhanced dopamine activity in the stratum of female rats, male rats were not affected. These findings may at least partly explain the higher risk of developing addiction to cocaine in females as well as the effect of the menstrual cycle on addiction behavior. The elevation of allopregnanolone levels in the cerebral cortex by ethanol (494) may explain in part not only the hypnotic effect, but also mild anxiolysis that may contribute to the development of ethanol addiction.

F. Mineralocorticoids

Early work on the rapid action of aldosterone, the principal mineralocorticoid, has been reported occasion-
ally. Studies include clinical work on hemodynamics (233) and on Na\(^+\) efflux from erythrocytes (462). Two decades later, a dual effect on Na\(^+\)/H\(^+\) flux in rat tail arteries was found that consisted of an early, ouabain-insensitive effect occurring within 15 min and a delayed Na\(^+\)-K\(^+\)-ATPase-dependent part (323). The rapid action was not affected by actinomycin D and thus is likely to be nongenomic. As suggested by the two latter studies, other ion fluxes are modulated rapidly as well in different cell types. Our laboratory demonstrated increases of [Na\(^+\)]\(_i\), [K\(^+\)]\(_i\), and [Ca\(^{2+}\)]\(_i\) in human mononuclear lymphocytes upon aldosterone stimulation that were half-maximal at 0.1 nM (for review, see Refs. 144, 519). The increase in cell volume that accompanies ion fluxes has been reported in both HML and endothelial cells (427), thus suggesting a more general role for cell volume regulation. Rises in [Ca\(^{2+}\)]\(_i\) have been demonstrated in many different cell types by our laboratory; an instructive example is shown in Figure 8.

The Na\(^+\) influx is probably induced by a rapid activation of the membrane-bound NHE, which also causes a pH rise (alkalinization) in the cell. This phenomenon has also been demonstrated by BCECF spectrofluorometry (517) in VSMC. Similar results have been reported recently in strips of human chorionic and uterine arteries, where alkalinization occurred within a few seconds (5). This work also reports instructive studies on agonists and antagonists (discussed in sect. II C2b). Cortisol was active as an agonist only after addition of the hydroxysteroid dehydrogenase inhibitor carbenoxolone. The rise in pH probably reflects activation of the NHE, as judged by the ability of amiloride derivatives to abolish the response.

Rapid aldosterone-induced changes of pH have been reported in the distal tubule of toad kidney (345). The intracellular alkalinization was amiloride sensitive and thus probably mediated by the NHE; however, the time frame of 15–20 min does not rule out genomic action. The same group reported a rapid modulation of plasma membrane H\(^+\) conductance (173) and activation of the ERK pathway (172) that subsequently affects NHE activation in Madin-Darby kidney cells (344). In the same system, nuclear signaling initiated by exposure to aldosterone has been studied. Atomic force microscopy was used in this study to detect the contraction of nuclear pore complexes in response to a Ca\(^{2+}\) signal, that in turn is known to be elicited by aldosterone. Furthermore, the shrinkage of nuclei containing fluorescently stained DNA within 2 min upon addition of aldosterone has been demonstrated; however, there were two different patterns of response. The author concluded that Ca\(^{2+}\) accumulated in the nucleoplasm at high concentration during contraction, thereby preparing the pores to specifically import the activated (ligand-bearing) MR into the nucleus, which eventually leads to genomic action. This model therefore integrates nongenomic and genomic action into a dual-action model. Recently, the rapid responses of skeletal muscle cells to aldosterone at 10–100 nM (besides testosterone) were studied (133). In this system, aldosterone induced a rapid transient rise in intracellular Ca\(^{2+}\) which peaks at 3 min, followed by a second slow increase being maximal at 30 min after hormone addition. The fast response occurs as a series of rapid oscillations in Ca\(^{2+}\) concentration on the single-cell level. Interestingly, Ca\(^{2+}\) oscillations distinct from the events in the cytosol occur in the nuclei, which may have physiological significance for muscle function. A transient increase of IP\(_3\) precedes the Ca\(^{2+}\) peak. The Ca\(^{2+}\) response remained unchanged upon preincubation with 1 mM spironolactone, and estradiol, progesterone, and dexamethasone were unable to elicit a response even at 1 mM.

In another recent study, nongenomic aldosterone effects on cortical collecting duct cells have been investigated (205). In these cells, aldosterone at 1 nM produced a transient increase of intracellular Ca\(^{2+}\) within 5 min.
Again, spironolactone was ineffective at 10,000-fold excess as was actinomycin D. The same laboratory suggested PKCα as a direct target for nongenomic aldosterone action (203, 204). In this scenario, PKCα activation leads to Ca\(^{2+}\) influx. The increased intracellular Ca\(^{2+}\) concentration together with a direct PKCα effect activates the NHE, which leads to alkalinization of the cell. This in turn stimulates K\(_{ATP}\) channels. The release of arachidonic acid by phospholipase A\(_2\) action and subsequent PGE\(_2\) formation is also thought to participate in mediating Ca\(^{2+}\) entry (204). The physiological meaning of these phenomena in Na\(^+\)-absorbing epithelia may be the maintenance of a sufficient ion potential to promote Na\(^+\) entry by activation of basolateral K\(^+\) conductance (202, 203). Although PKA was not found to be activated in distal colon cells (202), in bronchial epithelium PKA seems to participate in aldosterone signaling, as well as adenylate cyclase, Ca\(^{2+}\)-ATPase, and a G protein-coupled receptor (492). In contrast to many other systems, however, aldosterone leads to a decrease of intracellular Ca\(^{2+}\) in these cells.

In vitro studies on rapid aldosterone action frequently utilize steroid concentrations that are significantly above the levels found in humans at rest. However, under certain conditions such as physical exercise, aldosterone may reach nanomolar levels (478). Therefore, the physiological relevance of many phenomena reported in vitro can be assumed. Furthermore, local aldosterone synthesis has been demonstrated in tissue, such as the heart (450), thus posing the possibility of much higher local concentration. This is particularly important for cardiovascular pathophysiology as discussed below.

Although the quick response of plasma aldosterone levels to posture changes is well known, in vivo studies in search for rapid effects corresponding to these changes have been conducted only with lingering. Aldosterone has been shown to significantly facilitate phosphocreatinine recovery in human calf muscle after physical exercise within minutes after intravenous administration (530). The effect depends on participation of the anaerobic pathway, being absent when aerobic metabolism is used alone. In the dog, convincing in vivo evidence for rapid aldosterone action in vivo has been found for effects on baroreceptor neuron discharge frequency (510), where effects are detectable as early as 15 min after steroid administration. These findings were confirmed in humans in a clinical study, where aldosterone was found to blunt the baroreflex response (536). High aldosterone levels may therefore impair baroreflex function in hypertension or heart failure. The above-mentioned early studies done by Klein and Henk (233) have been confirmed in our laboratory using invasive techniques. Within 3 min after intravenous administration of 0.5 mg aldosterone, the systemic vascular resistance (SVR) increased (520) but returned to or even declined below baseline after 10 min. A modified follow-up study revealed changes in SVR during the first 45 min after steroid administration (425).

After exhaustive exercise, aldosterone was found to be increased with the level positively correlated to work load, possibly reflecting increased volume demand. However, physical training, which leads to a substantial gain in working capacity, does not affect aldosterone levels at rest or at exercise (175). A possible explanation is the suppression of the renin-angiotensin-aldosterone system (RAAS) secondary to training to counteract stress-induced activation (211). The action on baroreflex mentioned above and the reducing effect on heart rate variability (271) may point to an adrenergic-like action of aldosterone. A direct interaction has been shown as well between aldosterone and modulators of the α\(_1\)- and β-adrenergic system (424).

Increased mortality in patients with chronic heart failure or coronary heart disease has been correlated with activation of the RAAS. Downregulation of the latter or of the adrenergic system has both been successful lowering mortality in such patients. While angiotension-converting enzyme inhibitors are an established part of therapy, administration of the MR antagonist spironolactone has proven beneficial only recently during the Randomized Aldactone Evaluation Study (RALES) (378). While being an antagonist at the classic MR, spironolactone itself as well as its closed-ring metabolites form an equilibrium with their open-ring derivatives (see sect. 2C.2a) in vivo. Based on findings reported by Alzamora et al. (5), some open-ring derivatives appear to have antagonist activity for nongenomic aldosterone action in vitro, although more comprehensive data are not available. As the dose (25 mg daily) was much lower than usually employed to effect diuresis, the therapeutic benefit may have arisen, at least in part, by the inhibition of nongenomic mechanisms.

A different mechanism for the action of spironolactone has been proposed by recent findings in the isolated working rat heart (29). Here, spironolactone exerts an autonomous positive inotropic action and does not antagonize but adds to the inotropic action of aldosterone, with spironolactone being roughly 10 times more potent than aldosterone. This might explain much of the beneficial action of the antagonist seen in the RALES study.

G. Vitamin D\(_3\)

Starting from early observations in intestinal epithelia, rapid effects of 1α,25(OH)\(_2\)D\(_3\) on transepithelial movements of calcium have been termed "transcalcachnia." For rapid effects of vitamin D\(_3\) on intracellular calcium, both release from intracellular stores and influx have been identified as sources, with their relative contributions depending on the cell type studied (333). In chicken myo-
blasts, IP₃ and DAG have been found to be involved in triggering the calcium response (315), while rapid cGMP responses have been seen in human fibroblasts (30). Moreover, 1α,25(OH)₂D₃ (10⁻⁸ M) significantly increased MAPK phosphorylation, with the earliest response being detectable at 30 s (460). None of these immediate effects requires gene transcription or protein synthesis (145).

In this section we emphasize the signaling pathway and point out the possibly related clinical implications.

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 1α,25(OH)₂D₃ have been intensively studied in recent years. An increase in [Ca²⁺]i occurring within minutes in response to treatment with 1α,25(OH)₂D₃ (10–100 pM) has been found in primary cultures of mouse osteoblasts (254). Similar results have been described in rat osteosarcoma cells (ROS 17/2.8; Ref. 82 and others). Baran et al. (25) found an increase in [Ca²⁺]i accompanied the increased expression of osteocalcin mRNA steady-state levels in rat osteosarcoma cells. In a subsequent experiment they inhibited the rapid increase in [Ca²⁺]i by the inactive epimer 1β,25(OH)₂D₃ and found a reduction of osteocalcin mRNA. The results demonstrate the functional importance of the rapid, nongenomic actions of 1α,25(OH)₂D₃ in the genomic activation of the osteocalcin gene by the hormone in rat osteoblast-like cells (25).

Further studies on the rapid 1α,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 (82, 254).

A rapid increase in [Ca²⁺]i following 1α,25(OH)₂D₃ stimulation has been described in several other cells or tissues not commonly addressed as vitamin D target tissues. For example, in skeletal muscle cells (499) and in both cardiac muscle tissue and cultured chick cardiac muscle cells, physiologically relevant doses of 1α,25(OH)₂D₃ have been found to induce an increase of [Ca²⁺]i within a few minutes, an effect which was independent of mRNA or protein synthesis. Similar to osteoblasts, this effect seems to involve a voltage-dependent Ca²⁺ channel (112, 435). The clinical implication of this calcium increase, e.g., possible changes in electrophysiological parameters, remains to be clarified.

In addition to the activation of Ca²⁺ influx, 1α,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, 1α,25(OH)₂D₃ (100 pM) transiently increases [Ca²⁺]i within 5 s via IP₃ formation, an effect involving PLCα (250). Moreover, rapid, 1α,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 (505, 506). Similar 1α,25(OH)₂D₃-induced effects on the generation of DAG and IP₃ have also been found in a variety of other cells including typical vitamin D effector cells like enterocytes (64), dispersed porcine parathyroid cells (254), osteoblasts (461), ROS 17/2.8 cells (102), and keratinocytes (476), but also in cells like primary chick-embryo muscle cells (315) and hepatocytes (23). In rat skeletal muscle, the involvement of PLC and a PTX-sensitive G protein in the rapid 1α,25(OH)₂D₃-induced release of IP₃/inositol phosphates and DAG has been shown (137, 314). In addition, 1α,25(OH)₂D₃ was found to rapidly activate phospholipase D in Caco-2 cells (a human colon cancer cell line) (230), chick myoblasts (315), and rat skeletal muscle (138).

Because DAG can cause the activation of PKC, the influence of 1α,25(OH)₂D₃ on this protein has also been examined in several tissues and cells, respectively. In growth zone chondrocytes, 1α,25(OH)₂D₃ (10 nM) causes a rapid increase in PKC activity that was insensitive to actinomycin D and cycloheximide (67, 473). A recent study showed that the activation of PKC by 1α,25(OH)₂D₃ and 24R,25(OH)₂D₃ involves rapid increases in diacylglycerol via a phospholipase D (PLD)-dependent mechanism. 24R,25(OH)₂D₃, but not 24S,25(OH)₂D₃ or 1α,25(OH)₂D₃, stimulated PLD activity in resting zone cells within 3 min via nongenomic mechanisms. Neither 1α,25(OH)₂D₃ nor 24R,25(OH)₂D₃ affected PLD in growth zone cells. Inhibition of PI 3-kinase, PKC, PI-specific PLC (PI-PLC), and phosphatidylinositol (PC)-specific PLC (PC-PLC) had no effect on PLD activity (472).

Analogs of 1α,25(OH)₂D₃, which exhibit <0.1% of the binding to the classic receptor found for the parent compound, elicit comparable increases in PKC specific activity (188). In addition, a rapid activation of PKC by 1α,25(OH)₂D₃ has been reported in rat epithelium cells (506). Also, 1α,25(OH)₂D₃ has been shown to directly activate PKC, indicating the possibility of a 1α,25(OH)₂D₃ binding domain on the enzyme (455). A recent study on Ca²⁺ mobilization from inner stores and extracellular Ca²⁺ entry in chick skeletal muscle cells after stimulation with 1α,25(OH)₂D₃ could show that these effects were abolished by the PKC inhibitors bisindolylmaleimide and calphostin. The authors presume that 1α,25(OH)₂D₃ activates and translocates PKCα to the membrane, suggesting that this isozyme accounts for PKC-dependent 1α,25(OH)₂D₃ modulation of Ca²⁺ entry. With the help of antisense technology, the expression of PKCα was selectively knocked out by intranuclear microinjection of an antisense oligonucleotide against PKCα mRNA and the authors could demonstrate a reduction of the Ca²⁺ influx component of the response to 1α,25(OH)₂D₃, These results pointed out that 1α,25(OH)₂D₃ induced activation of PKCα enhances extracellular Ca²⁺ entry, partially contributing to mainte-
nance of the sustained phase of the Ca\(^{2+}\) response to the sterol (85).

Because PKC activation is linked to the activation of MAPK, the influence of 1\(a,25(\text{OH})_2\text{D}_3\) on this signaling protein was analyzed. MAPK is known to be able to integrate multiple intracellular signals transmitted by various second messengers so as to regulate many cellular functions by phosphorylation of numbers of cytoplasm kinases and nuclear transcription factors including the epidermal growth factor receptor, c-Myc, and c-Jun. Recently, it was shown that 1\(a,25(\text{OH})_2\text{D}_3\) is able to stimulate both Raf and MAPK pathways in keratinocytes (180, 280), chick enterocytes (114), and NB4 cells (460), indicating that the rapid response signal transduction components of 1\(a,25(\text{OH})_2\text{D}_3\) may be involved in the regulation of cell growth. In NB4 cells, 6-cis locked analogs of 1\(a,25(\text{OH})_2\text{D}_3\) (10 nM) increased MAPK phosphorylation with the same efficacy as 1\(a,25(\text{OH})_2\text{D}_3\) itself. The translocked analogs were inactive, and 1\(\beta,25(\text{OH})_2\text{D}_3\) had antagonist-like properties (460). Because in other systems the MAPK 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\(a,25(\text{OH})_2\text{D}_3\)-induced MAPK activation may represent a form of cross-talk that could modulate the genomic pathways of 1\(a,25(\text{OH})_2\text{D}_3\).

Moreover, the participation of nongenomic actions of 1\(a,25(\text{OH})_2\text{D}_3\) in the cAMP/PKA signaling pathway was analyzed. Several studies indicated that the rapid stimulation of Ca\(^{2+}\) influx by 1\(a,25(\text{OH})_2\text{D}_3\) in various cells was abolished by specific inhibitors of adenylate cyclase and PKA (113, 282, 498). In vitamin D-deficient chick soleus muscles, 1\(a,25(\text{OH})_2\text{D}_3\) (10 pM to 10 nM) elevated tissue cAMP levels within 45 s to 5 min and increased adenylate cyclase activity (150). Similarly, intracellular cAMP levels rose in chick cardiac muscle and rat enterocytes after stimulation with 1\(a,25(\text{OH})_2\text{D}_3\) (283, 434).

Aside from the effects described above, 1\(a,25(\text{OH})_2\text{D}_3\) has also been shown to elicit various other rapid nongenomic responses. Electrophysiological studies were done in ROS 17/2.8 cells where 1\(a,25(\text{OH})_2\text{D}_3\) but not 1\(\beta,25(\text{OH})_2\text{D}_3\) promoted the rapid enhancement of Cl\(^-\) current in a concentration-dependent manner (538). The hormone has also been reported to be involved in the stimulation of alkaline phosphatase activity (43) and phosphate transport (226, 471).

Moreover, 1\(a,25(\text{OH})_2\text{D}_3\) 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 by an accumulation of cGMP (30). Edelman et al. (124) described a rapid change (within seconds) in the membrane potential of the renal proximal tubule from the amphibian Neocallius resulting from the modification of Ca\(^{2+}\)-dependent K\(^+\) channels by 1\(a,25(\text{OH})_2\text{D}_3\) (124). Furthermore, an inhibition of renal 25(\(\text{OH})_2\text{D}_{27,1}\)-1-hydroxylase induced by 1\(a,25(\text{OH})_2\text{D}_3\) was found (118).

In a pancreatic β-cell line, 1\(a,25(\text{OH})_2\text{D}_3\) induces oscillations of [Ca\(^{2+}\)]\(_i\) mediated through nonselective calcium channels that are blocked by lanthanum ions (438). Fluorescent digital ratio metric video imaging at the single-cell level was used to study the effects of 1\(a,25(\text{OH})_2\text{D}_3\) on [Ca\(^{2+}\)]\(_i\) in a pancreatic β-cell line, RIN1046–38. In these cells equilibrated at a steady-state glucose concentration of 5.5 mM, 1\(a,25(\text{OH})_2\text{D}_3\) (2–20 nM) rapidly, within 5–10 s, increased [Ca\(^{2+}\)]\(_i\) and evoked sinusoidal [Ca\(^{2+}\)] oscillations. The [Ca\(^{2+}\)] oscillations were acutely dependent on extracellular Ca\(^{2+}\), but not on extracellular glucose. The 1\(a,25(\text{OH})_2\text{D}_3\)-evoked [Ca\(^{2+}\)] oscillations were mediated by nonselective Ca\(^{2+}\) channels, which are permeable to Mn\(^{2+}\) and suppressed by extracellular La\(^{3+}\). Blockage of voltage-dependent Ca\(^{2+}\) channels by nifedipine significantly decreased the amplitude of the oscillations. Unlike its effect on the aldosterone-induced response of [Ca\(^{2+}\)]\(_i\) in VSMC, depletion of intracellular Ca\(^{2+}\) stores with thapsigargin did not affect the 1\(a,25(\text{OH})_2\text{D}_3\)-stimulated Ca\(^{2+}\) entry estimated by the fura 2 fluorescence method. This demonstrates that the hormone directly activates nonselective Ca\(^{2+}\) channels. The 1\(a,25(\text{OH})_2\text{D}_3\)-evoked increase in the Ca\(^{2+}\) influx appears to generate [Ca\(^{2+}\)] oscillations by triggering Ca\(^{2+}\) release through the ryanodine receptor/Ca\(^{2+}\) release channel, but not through activation of the IP\(_3\) receptor. These findings are in line with a role of a plasmalemmal vitamin D receptor coupled to the plasma membrane Ca\(^{2+}\) channels in mediating rapid effects of the hormone (438). They also show that rapid steroid effects may share major similarities with regard to effects, e.g., on [Ca\(^{2+}\)]\(_i\); however, details of signaling pathways involved in effects of different steroids in various tissues may be widely variable. A summary of some possible pathways for nongenomic vitamin D action, with emphasis on MAPK activation, is shown in Figure 9.

Although there are many studies on the effect of vitamin D on insulin secretion by pancreatic β-cells, the role of vitamin D in cell function is not yet clear. Kajikawa et al. (224) have demonstrated the insulinotropic effect of a vitamin D analog through nongenomic signal transduction. This result has not yet been evaluated in respect to its clinical relevance, but it is tempting to speculate about a specific interaction of vitamin D or its analogs with a putative membrane vitamin D receptor of pancreatic β-cells that may enhance insulin secretion without side effects of currently used drugs. Kajikawa et al. (224) could show in a recent study that the vitamin D analog, 1\(a,25(\text{OH})_2\text{D}_{27,1}\), enhanced high concentration of glucose-induced insulin release together with enhancing the Ca\(^{2+}\) influx in pancreatic β-cells within 15 min. In addition, the effect was completely abolished by the antagonist of nongenomic action [1\(\beta,25(\text{OH})_2\text{D}_3\)], strongly suggesting a nongenomic vitamin D effect on insulin re-

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lease (224). It remained unclear why augmentation of the 
\([\text{Ca}^{2+}]/\text{H}^{11001}\) rise induced by 1,25(\(\text{OH}\))\(_2\text{lumisterol}3\) was ob-
served only in the presence of a high concentration of
glucose. It may be due to the linkage of signal transduc-
tion of 1,25(\(\text{OH}\))\(_2\text{lumisterol}3\) to modulation of the voltage-
dependent \([\text{Ca}^{2+}]/\text{H}^{11001}\) channels by the intracellular glucose me-
tabolism. These striking experiments show the direct clin-
ical implications of rapid nongenomic effects of a vitamin
D analog.

H. \text{T}_3 \text{ and } \text{T}_4

Rapid nongenomic responses to thyroid hormones
may be divided into several categories. In addition to
modulation of ion fluxes and intracellular kinase activities
which are commonly seen with steroids, intracellular pro-
tein trafficking can be a target, along with the cytoskele-
ton, both being phenomena that are observed more rarely
with steroids.

Among the earliest reports of rapid thyroid effects, \text{T}_3 was
reported to increase the 2-deoxyglucose uptake in
chick embryo heart cells (433) and later also in various
tissues of the rat (430, 431). The effect occurred in the
lower nanomolar range and, in heart slices, was associ-
ated with a spike of intracellular \([\text{Ca}^{2+}]\) that was maximal
after 30 s and originated from intracellular stores, as
judged by its independence from extracellular \([\text{Ca}^{2+}]\).
Rapid increases in intracellular \([\text{Ca}^{2+}]\) caused by \text{T}_3 have
been described frequently (432 and others). In red blood
cells and other cell systems, \text{T}_4 and \text{T}_3 have been shown
to stimulate plasma membrane (110,408) and sarcoplas-
mic \([\text{Ca}^{2+}]\)-ATPase, thus increasing \([\text{Ca}^{2+}]/\text{H}^{11001}\) ef-
flux from the cytosol. The activation of \([\text{Ca}^{2+}]\)-ATPase(s) is probably not
a direct one (111) but is likely to be mediated by PKC,
which itself is activated by thyroid hormones (see sect.
\(\mu\text{D8}\)), but without any particular PKC isoform identi-
sed so far. The enhancement of PKC activity has been shown
to follow PLC action (261), which forms DAG from phos-
phatidylinositol. IP3, which is the second product in this
reaction, is known to liberate \([\text{Ca}^{2+}]\) from intracellular
stores (50). Recently, \text{T}_4 has been shown to initiate a dual
DAG liberating phospholipase pathway, involving PLC and
PLD sequentially in liver cells (227). Activation of
PLD apparently is regulated by PKC, as judged from its
sensitivity toward PKC inhibitors, thus indicating a pos-
sible feedback mechanism.

The cAMP-dependent PKA has also been shown to be
activated by \text{T}_4 in HeLa cells (261), and both PKA and
PKC activation are required for \text{T}_4 enhancement of inter-
feron-\(\gamma\)-induced antiviral activity.

Furthermore, the MAPK signal cascade may be driven
by \text{T}_4, e.g., via a putative GPCR (259), finally exerting

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**FIG. 9.** Schematic diagram of mitogen-activated protein kinase (MAPK) cascade activation. 1,25(\(\text{OH}\))\(_2\text{lumisterol}3\) may bind to one or more of three putative classes of membrane receptors, resulting in MAPK activation by different mechanisms: 1) through membrane receptors with intrinsic tyrosine kinase activity; 2) through membrane receptors without intrinsic tyrosine kinase activity, utilizing src; 3) to generate either membrane-associated Ras-GTP that phosphorylates Raf isoforms; 4) more indirectly through activation of PI 3-kinase or phospholipase C (PLC), linked to diacylglycerol (DAG) activation of protein kinase C (PKC) isoforms. All pathways ultimately lead to Raf phosphorylation and subsequent activation of the MAPK cascade. [From Norman et al. (340). Copyright 2001, with permission from Elsevier Science.]

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multiple effects, such as modulating the phosphorylation of STAT (signal transducer and activator of transcription) proteins (259, 260) and p53 as well as nuclear TRβ1 (448). The latter is an example of nongenomic modulation of a nuclear, i.e., genomically acting, receptor for the same hormone.

Other nongenomic thyroid hormone effects described include a rapid stimulation of the Na$^+/H^+$ antiporter in L6 myocytes, resulting in a dose-dependent alkalization of the cell (220), and the shortening of the action potential in hypothyroid ventricular myocytes by comparatively high levels of T3 (469). Interestingly, this action potential in hypothyroid ventricular myocytes by the late-phase may explain the T3-dependent sensitization of cycloheximide-sensitive. This adrenoreceptor modulation in a coagonist fashion, such as the GABA$\_\alpha$ and similar receptors in many nongenomic phenomena, and many receptors as discussed above, is a useful criterion for distinction of pathway and receptor. To obtain a desired physiological response, it is obviously sufficient to apply a drug that preferentially acts on one or the other pathway. Knowledge of the receptor involved is not necessary. This in turn, however, points to a shortcoming in the research done to date. In contrast to the classic, genomically acting receptors, hardly any selective agonists or antagonists for nongenomic steroid actions are known, and we still have some way to go before such drugs can be used in clinical practice. Probably the only clinical application of nongenomic steroid action that has gained some popularity were anesthetic steroids (Althesin and others, now withdrawn for side effects unrelated to the steroid). However, the RALES study (378) provided hints to a possible nongenomic inhibition effect that has been extensively but unconsciously used. As discussed in section III, spironolactone is metabolized to canrenone, which forms an equilibrium with its open-ring congener canrenoate.

The clinical impact of nongenomic thyroid hormone action has been investigated in humans in vivo. Intravenous application of T3 significantly increased cardiac output while decreasing systemic vascular resistance (SVR), while heart rate and blood pressure remained unaffected in heart failure patients with diminished free T3 plasma levels (199). Here the short time frame was incompatible with genomic mechanisms. Direct modulation of vascular regulation, independent from the endothelium, may account for decreased SVR (356). T3 may affect Na$^+$ currents in isolated ventricular myocytes possibly linked to positive inotropic changes (123). The concomitant modulation of Na$^+/Ca^{2+}$ exchange increases contractility (508). T3 treatment was also found to lower the prevalence of atrial fibrillation in artery bypass graft patients, an effect that may be explained by the rapid response of some voltage-gated K$^+$ channels to thyroid hormones (234, 417). An early nongenomic, cycloheximide-insensitive modulation of β-adrenoreceptor density by T3 has been seen in cultured embryonic cardiomyocytes (496), while the late-phase β-adrenoreceptor upregulation was cycloheximide sensitive. This adrenoreceptor modulation may explain the T3-dependent sensitization of β-adrenergic inotropy (507) by increasing β-adrenoreceptor signal transduction and cAMP production, whereas T3 alone does not affect cAMP (508).

I. Does Receptor Type Matter for Clinical Impact?

As described in the previous sections, much evidence has been accumulated for the involvement of nonclassic receptors in many nongenomic phenomena, and many proteins are now known to act as such. This particularly applies to many receptors that are modulated by steroids in a coagonist fashion, such as the GABA$\_\alpha$ and similar receptors. Despite these data, sometimes the existence of receptors beyond those mentioned before is still doubted. From the clinician’s point of view, the physiological and pathophysiological phenomena that can be influenced by administration of drugs is of utmost importance. The pharmacological profiles, defined as the relative potency of substances to evoke or antagonize hormone action, are usually different for nongenomic effects. This difference, in particular the insensitivity toward inhibitors of classic receptors as discussed above, is a useful criterion for other pathway. To obtain a desired physiological response, it is obviously sufficient to apply a drug that preferentially acts on one or the other pathway. Knowledge of the receptor involved is not necessary. This in turn, however, points to a shortcoming in the research done to date. In contrast to the classic, genomically acting receptors, hardly any selective agonists or antagonists for nongenomic steroid actions are known, and we still have some way to go before such drugs can be used in clinical practice. Probably the only clinical application of nongenomic steroid action that has gained some popularity were anesthetic steroids (Althesin and others, now withdrawn for side effects unrelated to the steroid). However, the RALES study (378) provided hints to a possible nongenomic inhibition effect that has been extensively but unconsciously used. As discussed in section III, spironolactone is metabolized to canrenone, which forms an equilibrium with its open-ring congener canrenoate.

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IV. INTERACTION OF NONGENOMIC AND GENOMIC PATHWAYS

Despite the many differences between genomic and nongenomic pathways that have been laid out in the previous chapters, there are several points where interaction between each other might occur. Steroids have been shown to rapidly modulate the levels of intracellular cAMP. Recently, steroid-induced transcription of genes has been reported to be influenced by changes of cAMP levels (258, 336, 407). Integrating these findings into one concept, a two-step model of steroid action has been proposed (100, 516). In this model, originally developed for aldosterone but expandable to other steroids, the rapid nongenomic as well as the delayed genomic steroid action are comprised together with possible mechanisms of modulation of classic receptor-induced gene transcription by nongenomic signal transduction pathways (Fig. 10).
One of the most important universal signals in terms of cross-talk seems to be cAMP, the formation of which has been shown to be rapidly increased by steroids. This second messenger, formed from ATP by activated adenylyl cyclase, in turn activates PKA. The latter directly phosphorylates CREB and is thought to activate the MAPK pathway, presumably leading to SRC1 phosphorylation. The phosphorylated forms pCREB and pSRC1 co-operate in coactivation of steroid receptors as reported for the chicken PR isoform A (407).

An outline of a rather complex, additive, and maybe even synergistic interaction between nongenomic and genomic effects on estrogen-induced gene transcription has been reported recently in a neuroblastoma cell line (497). With the use of a two-pulse stimulation schedule, the early membrane effect elicited with the first pulse was found to be mandatory for full transcriptional activation by the subsequent estrogen pulse. Specific inhibitors indicated that PKA and PKC participate in the nongenomic part, whereas Ca$^{2+}$ seems to be involved in both the nongenomic and the genomic events.

The amphibian homolog of the mammalian PR, termed XPR, was found to be localized at cell membranes of *Xenopus* oocytes as its 82 kDa form in small amounts (14). Progesterone stimulation caused XPR to associate with PI3-kinase activity, and, subsequently, with p42MAPK. Considering the in vitro ability of the latter to phosphorylate the 110-kDa form of XPR, regulation by the nongenomic p42MAPK pathway may be hypothesized.

In addition, the human PR was shown to possess a polyproline motif that interacts in a ligand-dependent way with Src homology 3 (SH3) domains of various intracellular proteins (61). Thus PR, upon binding the agonist R 5020, can activate Src tyrosine kinase, which in turn may drive the MAPK pathway. Ultimately, MAPKs alter transcription factors in the nucleus, thus modulating the expression of genes not connected to steroid hormone responsive elements.

The latter two studies suggest a dual role for classic steroid receptors, modulating gene expression through signal cascades in addition to direct transcriptional activity. However, at least in the case of *Xenopus*, XPR does probably not explain all steroid effects on maturation, as other steroids such as RU 486, cortisol, and deoxycorticosterone, though inducing maturation, do not activate the XPR pathway.

V. SYNOPSIS: WHAT DO WE KNOW ABOUT NONCLASSIC RECEPTORS?

From the evidence discussed in the previous sections, nongenomic actions of steroid hormones appear to be mediated by three different mechanisms.
First, a "side effect" of the well-known classic steroid receptors is discussed, as convincingly demonstrated for some effects of estradiol and progesterone. In this context, the ligand selectivity profile remains largely identical for genomic and much of the nongenomic action according to current knowledge.

The second mechanism relates to nonspecific, direct effects at the membrane level. This mechanism may at least participate in the rapid, nongenomic action of glucocorticoids, because these steroids occur at rather high concentration. The selectivity pattern differs significantly between genomic and nongenomic action (78). However, no direct experimental proof has been made to link the steroids' effects on membrane fluidity to intracellular signaling. A recent study has shown significant influences of rather high steroid concentration on the fluidity of artificial bilayers (524), but despite the divergent fluidity effects, all hormonal steroids tested activated the model enzyme Ca$^{2+}$-ATPase, which better correlated to the mobility (increased in the presence of steroids) of the protein itself in the lipid bilayer than to the lipid mobility itself, without apparent steroid specificity. On the other hand, spin-labeled surface proteins on murine spermatozoa exhibited decreased mobility upon addition of progesterone (387), but no other steroids were investigated. Obviously, such data that measure bulk behavior of either lipid or protein do not closely correlate to physiological phenomena so far.

The third mechanism involves novel or nonclassic receptors that are not closely related to the classic receptors. There are several properties that differ considerably between the current knowledge about classic receptors and the experimental data obtained about many rapid, nongenomic effects. Classic receptors are thought to be soluble proteins located in the cytosol or in the nucleus. Many experiments, however, point to the existence of membrane intrinsic receptors for some steroids. Antibodies directed against the ligand binding domains of classic receptors sometimes stain cell membranes or detect bands in Western blots of membrane preparations, in the latter case often with considerably lower molecular weight. Because the use of antibodies always carries the risk of cross-reaction, additional confirmation is needed. Some very recent studies have demonstrated membrane location of classic receptors (14).

Some experimental approaches used for the identification of membrane-bound steroid receptors or steroid binding proteins may have led to confusion. Many different steroid derivatives have been used, either radioactively or fluorescently labeled or bearing photoaffinity moieties. In the steroid skeleton, the positions 3, 6/7, 11, 17 and 21 (if present) are easily derivatized. Sometimes, derivatives substituted at different positions are implied to be interchangeable, which is most probably not true. Steroids conjugated to proteins or other high-molecular-weight carriers have been used very often to demonstrate the presence of specific steroid receptors on the solvent-accessible membrane. Although this procedure is straightforward and convincing, there are two shortcomings: first, conjugates inherently may contain free, unconjugated steroid that has to be removed (131). Some commercial preparations are not even labeled as to whether they contain free steroid. Often, the potency of steroid conjugates to evoke effects is 30- to 100-fold lower than free steroid, which correlates well with the amount of free steroid present in many preparations (1–3%). Second, recent reports have shown that the presumably cell-impermeable conjugates have been detected inside the cell (307, 335), so physiological actions exerted by steroid conjugates pose some difficulties for interpretation. However, studies that demonstrate exclusive membrane localization of fluorescently labeled steroid conjugates [e.g., confocal techniques (44)] still give strong evidence for steroid receptors located in the membrane.

The identification of binding sites for a specific steroid in a biological membrane does not necessarily imply any receptor property in the sense of signaling. There are many proteins known that bind steroids in a more or less specific way, often just due to the steroids' hydrophobicity. Even if a steroid initiates a signaling cascade involving second messengers, there is not a compulsory link to membrane binding, as long as the putative receptor is not identified and available for investigation. However, some examples still strongly suggest a functional connection, such as the demonstration of interference, in both binding and function with other receptor systems, e.g., the γ-adrenergic receptor (326), which unfortunately is poorly understood itself yet, or the GABA receptors in the nervous system (410).

From the view of physiology, many studies in search for nongenomic steroid effects use synthetic, nonphysiological steroids. A popular example is dexamethasone, a fluorinated glucocorticoid exhibiting ~30-fold potency, compared with the physiological ligand cortisol, as measured by classic genomically mediated responses. It has been shown, however, that the potency ranking for nonclassic effects is markedly different (78). This is also true for the progesterone effects on spermatozoa, which hardly correlate with affinities toward the classic cytosolic PR as convincingly demonstrated by Blackmore et al. (59) for a very large set of substances. If there are proteins, unrelated to classic receptors, mediating nonclassic responses, it is fair to assume that they may have a different selectivity pattern for their ligands. Instead of, or at least in addition to, using analogs that have been selected for their high potency to genomic receptors and responses, employing the physiological hormones could contribute to avoid false-negative results.

In summary, there is a considerable body of information about physiological events on the cellular level initi-
Nongenomic effects present in knock-out mice (MR, ER)
Pharmacological profile for agonists very different to classic receptors
Some compounds inhibit nongenomic responses despite very low affinity to classic receptors (MR, VDR)
Antagonists at classic receptors have no effect on nongenomic action (MR, PR)
Allosteric modulation of many receptor systems by steroids demonstrated (GABA, NMDA, sigma, Maxi-K channel, \(\gamma\)-adrenergic receptors)
The brassinosteroid receptor in plants, not related to vertebrate classic steroid receptors
Receptors for steroid phenornenes, either in the olfactory or vomeronasal organ (reported to be cloned)

VDR, vitamin D receptor; ER, estrogen receptor; AR, androgen receptor; PR, progesterone receptor; MR, mineralocorticoid receptor; NMDA, N-methyl-D-aspartate.

ated by steroids on the nongenomic pathway. The wide variety of cells and tissues in which such effects have been demonstrated show that it is a universal phenomenon rather than a rare object. Some receptors for nongenomic steroid action are well-known proteins, e.g., the receptors for neurosteroids. On the other hand, little is known about the nonclassic receptors that stand at the very beginning of most events, and the controversy that has been outlined in the previous sections is not yet settled. Some evidence for and against the participation of classic and nonclassic receptors is summarized in Table 1.

The clinical implication and relevance of nongenomic steroid action is occasionally glittering through the experimental data; however, its thorough investigation and exploitation is hampered by the lack of selective agonists and inhibitors for nongenomic action, with the exception of vitamin D. Phenomena such as rapid glucocorticoid action in shock, anesthetic and other neurotropic steroids, progesterone-induced acrosome reaction and, possibly, aldosterone-mediated aggravation of cardiovascular disease are fields that will probably be worth-while for closer investigation.

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