# MINIREVIEW

# Proteins of Multiple Classes May Participate in Nongenomic Steroid Actions

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**Responses to steroids initiated from non-nuclear receptors impinge on a wide variety of cellular responses and utilize nearly all known signal transduction webs. While the mechanisms by which steroid receptors localize in the membrane are still unclear, it is apparent that this alternative localization allows steroid receptors to participate in a wide range of complex functions influencing cell proliferation, death, and differentiation. The central debate still remains the identity of the protein class or classes that mediate membrane-initiated (nongenomic) responses. The data thus far have supported several possibilities, including: nuclear steroid receptor-like forms in non-nuclear locations; other known (nonsteroid) membrane receptors or channels with additional steroid-binding sites; enzymes; transporters; receptors for serum steroid-binding proteins; unique and previously undescribed proteins; or chimeras of typical steroid receptor domains with other unique or known protein domains. Categorizing membrane steroid receptor proteins based exclusively on the actions of antagonists and agonists, without considering cell context and protein partnering issues, may mislead us into predicting more receptor subtypes than really exist. However, the plethora of signaling and functional outcomes may indicate the participation of more than one kind of steroid-binding protein. Resolving such unanswered questions will require future investigative focus on this alternative arm of steroid action, which is likely to yield as many therapeutic opportunities as have nuclear steroid mechanisms.** Exp Biol Med 228:1272–1281, 2003

**Key words:** protein partners; receptors; steroid-binding proteins; protein kinases; signaling; subcellular localization

## Importance of Membrane-Initiated (Nongenomic) Steroid Actions in the Scheme of Things

The cellular strategies for responding to steroids include several types of mechanisms. Rapid responses can initiate second messenger-triggered signal cascades. Intermediate time scale actions can occur where the rapid activation of a molecule may lead to the gradual build-up of other response molecules (e.g., enzymatic conversion of substrates to products). Macromolecule-synthesizing (genomic) responses require investing many cellular resources in remodeling the cell's protein repertoire (and sometimes cell number), and are much slower. Over the past 30 years most studies have focused on this later, genomic phase of steroid responses. However, an increasing number of recent studies have described initial, rapid responses in more detail. We now know of examples of these initial responses for almost every class of steroid and related compounds (1, 2). It is thus increasingly clear that membrane-initiated (rapid) steroid responses are a general feature of the action of steroids that are very important to study if we are to understand the overall patterns of steroid actions. Understanding this widely used cellular strategy and its regulation and ligand specificity will doubtless be relevant to many medically important problems and present promising and unique therapeutic opportunities. For example, specific new estrogenic compounds may enhance bone maintenance through membrane-initiated actions, while not causing inappropriate breast or uterine cell growth (3).

Other recent reviews have summarized the nature of nongenomic responses and their diversity. These appraisals have classified nongenomic actions on the basis of steroid class (2, 4) or signaling pathways utilized (1, 5), or have focused on tissue-specific examples (6). Our analysis will instead focus on a different aspect of this problem: summarizing the arguments for the singularity or multiplicity of

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proteins (and their identities) that mediate rapid or nongenomic steroid actions, and the implications of this organization for the overall cellular responses to steroids.

## General Features of Membrane Steroid Receptors: Some Clues to the Identities of These Proteins

Where membrane steroid receptors have been observed directly via fluorescence imaging with antibodies or ligands, they appear as mobile, punctate patches of varying sizes located asymmetrically on cells (7–12). The size of these receptor "clusters" and their implications for organization within membrane shells, rafts, and caveolae (13), are currently being debated in the literature. Such clustering in rafts could affect receptor activation and interactions with other signaling molecules (13–15). How do we explain the physical location of nuclear forms of steroid receptors in the plasma membrane, and does this require modifications of a single protein or an entirely different protein? Multiple laboratories have confirmed that membrane steroid receptors can be found in caveolae (14, 16–19). One line of evidence suggests that interactions with specific domains of the caveolar structural proteins (caveolins) drag membrane estrogen receptors to the plasma membrane (20). Other evidence has shown that acylation of these receptors causes them to be imbedded into the membrane, perhaps along with alternative splice variant products (21). Yet another possibility may involve co-expression of small peptides originating in alternative 5′UTRs (22). Finally, unique receptors have been identified for some steroids, and their structure predicts that they traverse the membrane seven times like typical G protein-coupled receptors (23, 24). Rather than concluding that these reports are conflicting, we should consider the possibility that multiple membrane localization mechanisms and receptor proteins can act simultaneously or sequentially, and in a cell and receptor context-specific manner.

Except for specialized cases in which the nuclear compartment is absent so steroid actions can easily be classified as nongenomic [(e.g., collagen matrix vesicles, platelets, red blood cells, and enucleated oocytes (25–27)], most cells that have membrane steroid receptors also have nuclear steroid receptors of the same class. Unless receptor subclass or cellular location-specific ligands have been demonstrated (28, 29), this has presented a problem for investigators trying to assign a specific role in particular functions to a membrane steroid receptor, and to prove that membrane assignments for nuclear proteins are not due to contamination artifacts. We have tried to address this question by selecting cell subpopulations enriched or depleted for the membrane receptor, which can be correlated to the enhancement or diminution of the corresponding response, respectively (7, 10, 30, 31). However, the multiple and simultaneous cellular localizations of steroid receptors (nuclear, membrane, cytosolic, and perhaps other) have also given rise to the idea that they are interconnected in a continuum of responses starting at the membrane and sometimes ending in the nucleus (32).

Many studies have shown the presence of membrane receptors in cells of immature developmental stage (33, 34), cancer cells (7, 35–37), or cells subject to steroid-induced proliferation (38, 39). Thus it is possible that membrane steroid receptors diversify by using developmentally controlled expression mechanisms such as different splice variants (21, 40). Perhaps localization in the cell membrane facilitates interactions with other cell surface proteins involved in sensing adjacent cell-derived signals that affect proliferation or migration. Membrane steroid receptors may also be in an appropriate location to directly influence the cell cycle machinery, as other membrane or cytoplasmic signal transducers do (41, 42). These features are critical for developmental purposes, but may also serve as markers for the assessment of tumor stage and hormone responsiveness. While these features of membrane steroid receptor expression suggest a role in cell stage-regulated function, this question is still largely unaddressed.

The ability of antibodies to specifically label, elicit responses through, or disrupt the activities of membrane steroid receptors has been used to identify the proteins responsible for steroid-induced nongenomic actions. However, the identification of steroid receptors in the membrane via antibody recognition is plagued by the need to optimize fixation conditions for maximal epitope recognition. It is equally important to avoid the permeabilization of cells, which would allow the more prevalent nuclear antigen to predominate and obscure the membrane labeling (7, 9, 43). Such conditions are unfortunately cell type-specific, presumably due to the unique lipid composition of particular cell membranes, and so must be worked out for each new cell type. Specific antibody blockage or triggering of nongenomic responses (44, 45) ties the epitope identification of the protein to a specific receptor-induced response.

The use of impeded ligands has provided additional proof for the existence of membrane (vs. nuclear) receptors involved in specific responses. The nature of these reagents renders the conjugated steroids active only at the cell surface because they are incapable of entering the cell directly. Though the stability of such reagents has been called into question (46), these difficulties have often been overcome experimentally by removal of detached free steroids from the mixture immediately before use (47, 48). Interestingly, these impeded reagents are sometimes more potent than the corresponding concentration of free steroid, perhaps because of their prolonged residence at the membrane (due to an inability of the cell to process them away) or because of their multivalent nature (engaging and perhaps clustering several receptors together). Unfortunately, such ligands do not resolve the issue of the receptor's protein identity, only its membrane residence.

Membrane-initiated steroid responses sometimes exhibit a slightly different range and hierarchy of effective steroid concentrations than do genomic responses, although

still adhering to class-specific steroid-binding groups (reviewed in Ref. 1). Different steroid specificities have in the past been interpreted as evidence for a different receptor. Certainly, many new subclasses of receptors have been correctly predicted and subsequently cloned using this philosophy. But does a different ligand-binding profile always predict a unique receptor? Recent work indicates that differences in the binding characteristics of receptors might also result from a different cellular location or context. For example, the same estrogen receptor cDNA transfected into different recipient cell lines results in different cell type-specific ligand binding and responses for estrogens, xenoestrogens, or antiestrogens (49), including nongenomic responses (compare Ref. 50 to Ref. 51). This implies that components of the local environment contribute to a change in the ligand binding pocket of the receptor protein. Changes in agonist/antagonist binding with cell context could be due to the different repertoire of interacting proteins in different cell types. This is not surprising, as the conformations and functions of other proteins are often changed by protein–protein interactions. For example, nuclear receptor co-repressors can be changed into co-activators depending on other interacting proteins (52). Membrane steroid receptors are just as likely to interact with various modifying proteins (53). A change in the conformation of a membrane steroid receptor, and thus its ligand binding specificity, could also be due to the border lipids surrounding the protein in the membrane, as we know that chemical environment can alter a protein's substrate specificity (54).

Receptor-altering localizations and interactions could also lead to unanticipated results when nonendogenous mimetic compounds act as receptor ligands. When such compounds do not activate nuclear receptor reporter gene responses, such results could suggest that unique receptors mediate the xenobiotic responses. For example, environmental or dietary mimics of steroids (such as xenoestrogens or dietary estrogens) can bind to nuclear estrogen receptors only when their concentrations are high. Yet at far lower concentrations they may cause developmental disruption, possible carcinogenic consequences, or serve as beneficial estrogen replacement alternatives in humans (55) via currently unknown mechanistic pathways. When these estrogen mimetics are tested only for genomic effects, their ability to bind and activate membrane estrogen receptors may be overlooked.

Very high concentrations of steroids or their mimetics are reportedly necessary for some membrane-initiated effects (e.g., Ref. 56) and can lead to assumptions about receptor identity. In specialized steroid-producing tissues like ovaries and testis, these high concentrations may be relevant for physiological functions. However, it is unlikely that humans or animals would ever be exposed to such high concentrations in other tissues, either naturally or via accidental exposure. The requirement for high concentrations of steroids could also suggest that metabolites of the administered steroid are really the specific ligands for a given receptor and that the limited amount of metabolites produced can activate high-affinity receptors. Finally, it is possible that the high-concentration effects observed experimentally are not receptor-mediated, but may reflect an altered composition of membranes caused by steroids partitioning into them. The medical relevance of such studies depends upon whether such high concentrations can be achieved therapeutically, should that be warranted (e.g., for estrogenic protective effects against stroke in the brain (57)) without incurring unacceptable side effects.

## Multiple Class Identities of Proteins Mediating Nongenomic Steroid Actions

Given the significance of steroid action at the plasma membrane, it is very important to establish the identity of these signal initiators. The data thus far support a variety of receptor classes being responsible for membrane-initiated steroid actions, including: nuclear steroid receptor-like forms in non-nuclear locations; other known (nonsteroid) membrane receptors or channels containing additional steroid binding sites; enzymes; transporters; receptors for steroid-binding proteins of the serum; unique and previously undescribed proteins; or chimeras of typical steroid receptor domains with other unique or known protein domains (58). Figure 1 summarizes the general features of membrane steroid receptor subcellular localization, identities, and partnering. It shows a variety of signaling machineries that might be affected, either separately or simultaneously, by these juxtapositions.

The majority of steroid classes are now represented in the category of nuclear-style receptors in the membrane or other non-nuclear locations. Some studies have identified membrane or non-nuclear receptor proteins with specific antibodies and some of these reports additionally show direct interactions with other molecules that signal from the membrane (7, 9, 10, 19, 21, 27, 28, 34–36, 43, 59–73). Others reports show the specific diminution of membrane steroid receptors via antisense knockdown with nuclear receptor sequences (9, 33, 74). In contrast, others report that nuclear receptor antibodies do not recognize the corresponding membrane steroid receptor (see Ref. 75; unpublished reports). Such reports suggest that the receptors are entirely different proteins, or that not all epitopes are exposed in the membrane location (76); multiple antibodies were not tested in most of these studies.

Other membrane steroid receptors are apparently known proteins with other identities. These proteins are hypothesized to contain a steroid-binding site within their structures. Very diverse types of proteins have been described as fulfilling this role. For example, membrane glucocorticoid receptor has been identified as the κ opioid receptor (77). Receptors for neurosteroids, estrogens, and progestins have been identified by some as GABA or 5HT receptors (78–84). The membrane estrogen receptor has been identified as a γ-adrenergic receptor (85) or chloride

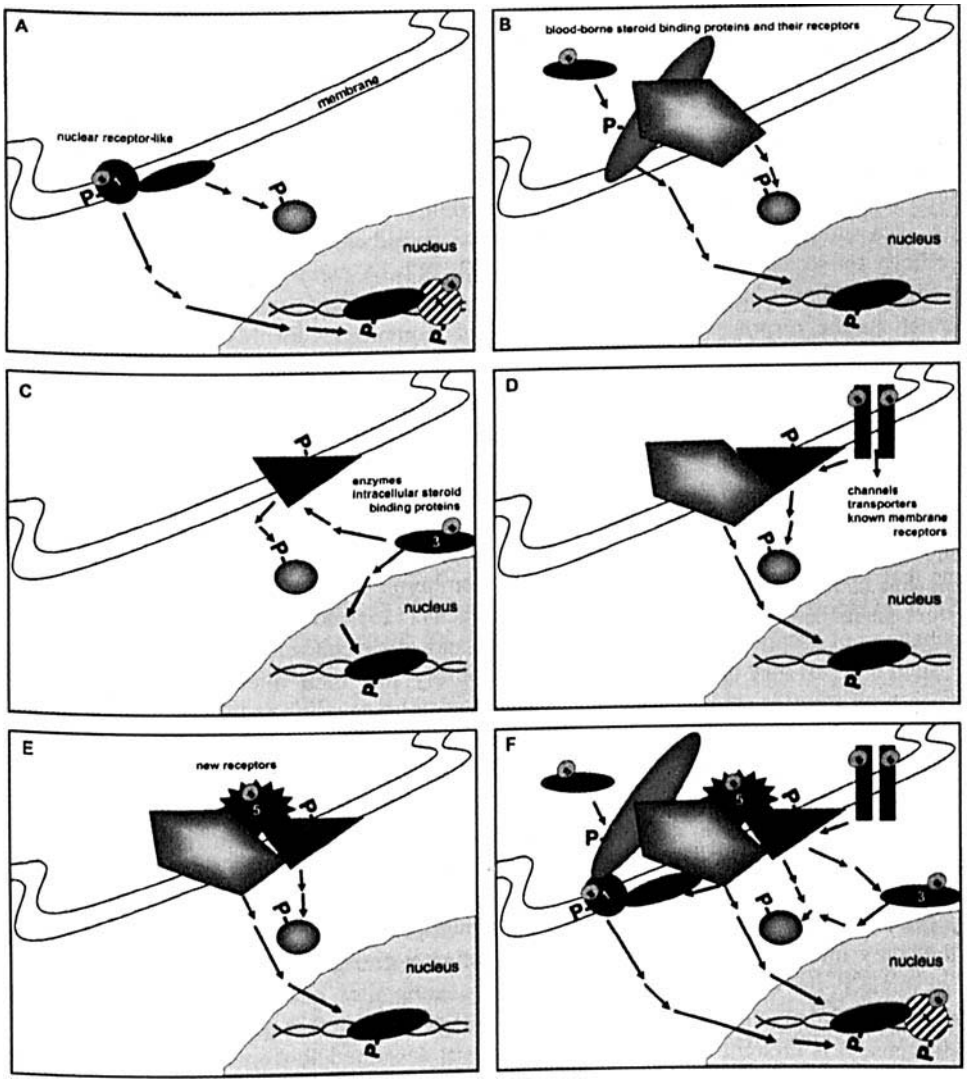


**Figure 1.** The possible heterogeneity in steroid-binding proteins involved in mediating nongenomic steroid actions is depicted hypothetically in these diagrams. Each lettered panel depicts a steroid receptor with a different protein class identity (symbols numbered 1–5). Steroid receptors or steroid-binding proteins also have a small circle labeled "S" (for steroid) attached to them. Alternate shapes depict different protein class identities, while alternate fill patterns depict different conformations of a single protein (e.g., 1 vs. 1-crosshatched for nuclear receptor versus nuclear receptor in the membrane in Panel A). Many of these different protein classes and their partners can be regulated by their phosphorylation status (shown by -P). Some signal cascades may culminate in the post-translational modification of transcription factors (symbols labeled "TF" in all panels, also see symbol 1-crosshatched in Panels A and F), making these effects ultimately genomic. Protein partners or targets of steroid receptors (unlabeled geometric shapes) can be located outside the cell, inside the cell, or in the membrane (all panels). The membrane indentation (all panels) refers to the residence of many signaling proteins, including membrane receptors, in membrane rafts or caveolae. For economy of design, some panels and symbols represent different kinds of proteins, as described below. Classes of proteins that bind steroids and participate in initially nongenomic responses could include: classical nuclear-type receptors in a membrane location (Panel A, Protein 1), blood-borne (Panel B, Protein 2), or intracellular (Panel C, Protein 3) steroid-binding proteins, enzymes (Panel C, Protein 3), transporters or channels or other known membrane receptors (Panel D, Protein 4), or entirely new receptors (Panel E, Protein 5). Multiple signaling pathways with numerous steps and directions (arrows) may convene on, and emanate from, these protein clusters, depending upon the cellular signaling context. These combinatorial effects can lead to response complexity. Shown in Panel F, multiple steroid-binding proteins may participate in signaling cascades leading to multiple functions in the same cell.

channel protein (86). This type of steroid action could occur via allosteric regulation. However, mutational analysis of these receptors has yet to pinpoint sequence locations that are responsible for steroid binding, as would be expected if a steroid binding pocket were an integral part of the protein. In many instances steroid effects on neurotransmitter responses were demonstrated in transfection systems where the participation of other interacting proteins present or recruited to the membrane (including steroid receptors) could not be ruled out. One example is the experimental expression of many of these neurotransmitter receptors in the *Xenopus* oocyte, which has long been known to have a membrane progesterone receptor (87, 88) that was recently determined in some studies to be a form of the nuclear progesterone receptor (27, 59). Steroid receptors could also modify other membrane receptors through the protein kinases that they activate (89). It remains to be seen if any of these examples will be further informed by investigations of steroid allostery versus steroid receptor partnering.

A few reports suggest that membrane steroid receptors are enzymes of known function. The steroid presumably binds to a site on the enzyme other than the substrate-binding pocket and allosterically changes the enzyme's activity. Examples are membrane estrogen and mineralocorticoid receptors identified as protein kinases (90), and membrane estrogen receptor reported to be an ATPase (91) or glyceraldehyde-3 phosphate dehydrogenase (92). Again, mutational analyses have yet to confirm these observations by indicating the steroid-binding portion of these enzymes. Alternatively, steroid receptors partnering with these proteins, either directly or indirectly via adaptors (93), may in some assays make it appear as if steroid binds to the enzyme

itself. In addition, a variety of steroid- metabolizing enzymes bind steroids, usually with lower affinity, but this low affinity could well explain some nongenomic actions that require micromolar steroid concentrations. There have been other cases where the identity of a new steroid receptor has been disputed, based on its possible identity as an enzyme. Such is the debate on the type II estrogen binding site. While some describe this site as a unique steroid-binding protein with distinctive specificity for steroidal and nonsteroidal estrogens (94), others conclude that it has all the features of an enzyme contributed to the responsive tissue (uterus or mammary gland) by eosinophil infiltration (95).

Lipophilic steroids have generally been reported to enter cells by partitioning into the plasma membrane and then escaping into the intracellular compartment, presumably because of their greater affinity for soluble intracellular receptors. However, several investigators have described specific plasma membrane transport proteins that facilitate cellular entry or exit of steroids (96–100). Such transport may function in some cases as a cellular mechanism of escape from steroid doses that can kill (30, 101). Such proteins may have been called receptors because they can bind steroids selectively, saturably, and as a result impinge on signaling pathways leading to proliferation, apoptosis, or other functions.

Some cells have plasma membrane receptors for blood-borne steroid-binding proteins, including testosterone-estrogen binding globulin [TEBG (102)], corticosterone-binding globulin [CBG (103)], and retinoid-binding protein [RBP (104)]. Because such proteins dock to cell membrane receptors, it has been speculated that they mediate membrane-initiated steroid actions. An example is the cell surface TEBG receptor, which could serve as a membrane receptor for both estrogens and androgens. It is thought to mediate cell proliferation responses in prostate cancer cells via a G protein-coupled pathway (102). In this context, a cell surface receptor for serum albumin could also be a membrane steroid receptor for all the steroids to which it binds (105). Such binding sites for the serum steroid binding proteins could be the membrane steroid receptor themselves, or could be intermediates in the delivery of steroids to separate membrane-resident steroid receptors. Clearly, the precise role of these binding protein receptors in initiating or facilitating nongenomic steroid effects must be further investigated.

Still other investigators contend that steroid receptors on the plasma membrane are unique, previously undescribed proteins. The most well-developed example involves membrane receptors for reproductive steroids, which regulate maturation of fish oocytes and the function of other reproductive tissues (106–109). Altogether, this research group has now identified candidate membrane receptors for progestins, androgens, and estrogens. All have the predicted structure of 7-transmembrane receptors, which couple to G-proteins, and have the corresponding antagonist profiles. With the advent of more complete sequence databases for multiple species, these proteins have been quickly related to likely homologs in multiple species, including human. Other investigators have cloned unique protein sequence candidates for membrane progestin and mineralocorticoid receptors, but their lack of homology to known protein classes has slowed the generation of testable hypotheses about their function (110, 111). Still other candidates for unique membrane steroid receptors have been described for estrogens (85, 92, 112). A membrane receptor for the vitamin D hormone has been biochemically characterized, but sequence and thus homologies for this unique smaller protein are still unavailable (113, 114).

Finally, chimeras representing combinations of the above categories have been suggested for the identity of other membrane steroid receptors. Some nuclear steroid receptor antibodies can recognize these proteins, and others cannot, which may suggest a protein composed of partial nuclear receptor sequences along with other protein domains. Such membrane steroid receptors have been described for estrogens (115) and progestins (116, 117). Their molecular sizes and other characteristics also suggest that they are not entirely like their nuclear receptor complements. Isolation of unique coding sequences will be necessary to understand whether these proteins truly represent a class of proteins sharing only some domains with nuclear receptors.

Are all these proteins different forms of membrane steroid receptors? The current prevalence of the "known protein" category (steroid receptor or other) may only be due to the fact that known proteins are easier to identify, characterize, and investigate, given the variety of existing experimental tools (e.g., antibodies, cDNAs) for their study. Only further study will reveal whether some or all of the alternative explanations described above persist. But we should also look for possible reconciliation of some of these identities. Could some of these protein types interact in the signal cascade machinery where the participation of more than one steroid-binding protein is necessary? Or do some of these proteins specifically interact with a nuclear receptor and sometimes give the appearance of imparting steroid-binding status to another protein? As we accumulate additional pieces of the puzzle, perhaps some of these examples will merge, incorporating the activities of several proteins into the same story.

## Do Diverse Proteins Mediate Steroid-Induced Actions, or Can One Style of Steroid Receptor Do Many Things?

We are learning that steroid actions initiated at the cell membrane can affect diverse and complicated processes in the cell: proliferation (37, 71), migration (118, 119), differentiation (119, 120), apoptosis (121), gene expression (5, 122), and overall coordination of other cell signaling events such as rapid release of hormones or neurotransmitters (7, 45, 123). Many of the more slowly developed responses are

now recognized as beginning with rapidly initiated steroid-induced signals. In fact, the terms genomic and nongenomic are difficult to apply, as we know that steroid signaling can cause rapid post-translational modification of transcription factors, which then initiate changes in gene expression that manifest themselves only as their products accumulate. Steroid-induced changes in neuronal cell inputs and outputs can result in rapid adjustment of the whole organism through behavioral changes. Signal-induced coordination of responses in cell cohorts often involve steroids, and may result in cell expansions (proliferation), contractions (apoptosis), and migrations, the hallmarks of developmental processes. These complex cellular responses can consist of many individual, simpler response components added together, or programmed in a temporal sequence. Because of this complexity, we may not yet fully understand where signaling pathways intersect and combine into response compendiums.

Our incomplete knowledge of the pathways for such very complex functions leaves room for considering functional diversity in resolving the above alternatives to membrane steroid receptor identity. Is it possible that more than one steroid-binding protein is necessary for nongenomic actions, or are some of the recent identifications just cases of mistaken identity? Have entirely different functional classes of steroid receptors evolved because of the divergent endpoints that they must serve, or the subtle differences in their roles?

However, single proteins can be quite diverse in their activities. Recent work has documented several instances of dual-protein identities. That is, enzymes have also been found to be transcription factors, comodulators, and binding proteins (124–126). Are these due to cellular economies, or do these aliases arise because of the need to coordinate several seemingly unrelated (but more likely misunderstood) processes? The interaction of growth factor/peptide hormone receptors and their effectors with steroid receptors may also contribute to the complexity of steroid signaling generated by a single protein. Steroids modulate membrane EGFR or Her-2 activation of Akt kinase (127). In one system, estrogen signaling to MAP kinase is dependent upon release of heparin-bound EGF to activate the EGFR (112). However, EGFR has also recently been shown to interact directly with the estrogen receptor-α (128). Additional interactions of steroid receptors with other membrane receptors have also been demonstrated (66, 72). Such interactions offer opportunities for determining how physical contacts of these membrane proteins (directly or though adaptors) are mechanistically involved in steroid responses. Thus membrane steroid receptors can be organizing centers for interactions, or comodulators of other functional proteins, depending upon your receptor-centric viewpoint. In any case, traditional steroid receptors are very versatile due to their ability to interact with other proteins.

## Do Single or Multiple Receptors Promote the Integration of Membrane and Nuclear Signaling?

Complex cellular responses are likely to start with membrane-initiated steroid action as the initial signal that reaches the cell from the circulation or adjacent cells. Decisions must eventually be reached as a result of the summing of multiple signals incoming from the surface, interacting with cellular "readiness" signals. These signaling summations "throw molecular switches" (typically, rapidly-acquired post-translational modifications such as phosphorylations) that alter the functions of pivotal proteins. The accumulation and integration of signaling events and protein modifications eventually results in more dramatic steps (synthesis of new macromolecules). Thus the two signaling pathways for steroids (i.e., membrane and nuclear) are probably intertwined. Is this because they are the same protein, or because distinct nuclear and membrane proteins interact with one another? Trying to specifically knock out the signaling from one receptor location can affect the other signaling mechanism. For instance, blocking estrogen-induced MAP kinase activation also knocks out PRL mRNA expression (129). Decreasing the synthesis of one form of the receptor (via antisense technologies) can also decrease the other form (9). Such results suggest, but do not necessarily prove, that the same receptor may function in both nuclear and membrane locations, and may even be part of interchangeable populations. However, more specific approaches to directly answering this question still elude us.

## Summary and Conclusions

There remains a critical need to compare the different examples and concepts about the nature of membrane steroid receptors to try to connect overlapping categories, or to more confidently resolve and accept multiple different categories of such proteins.

Do multiple divergent cellular requirements warrant these multiple proteins, which can be activated by steroid binding? Or do context-modified receptor systems explain the diversity of responses? We currently have too few examples, and no examples of membrane versions of the more newly discovered steroid receptor family members and orphan receptors, probably because they have not yet been investigated for this property. Perhaps their addition to these investigations will assist in the accumulation of sufficient examples to help us resolve these issues.

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