The interface of nuclear and membrane steroid signaling

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Abstract

Steroid hormones bind receptors in the cell nucleus and in the cell membrane. The most widely studied class of steroid hormone receptors are the nuclear receptors, named for their function as ligand-dependent transcription factors in the cell nucleus. Nuclear receptors, such as estrogen receptor alpha, can also be anchored to the plasma membrane, where they respond to steroids by activating signaling pathways independent of their function as transcription factors. Steroids can also bind integral membrane proteins, such as the G protein-coupled estrogen receptor. Membrane estrogen and progestin receptors have been cloned and characterized in vitro and influence the development and function of many organ systems. Membrane androgen receptors were cloned and characterized in vitro, but their function as androgen receptors in vivo is unresolved. We review the identity and function of membrane proteins that bind estrogens, progestins and androgens. We discuss evidence that membrane glucocorticoid and mineralocorticoid receptors exist, and whether glucocorticoid and mineralocorticoid nuclear receptors act at the cell membrane. In many cases, integral membrane steroid receptors act independently of nuclear steroid receptors, even though they may share a ligand.

KEYWORDS: nuclear hormone receptor, membrane signaling, non-genomic signaling, steroid signaling
**Introduction**

Steroids regulate gene expression in the nucleus but can also bind at or near plasma membranes and rapidly spark changes in cell physiology. When the nuclear receptors were cloned in the 1980s, the field focused on steroid signaling in the nucleus. In the last two decades, scientists learned more about how steroids signal at the cell membrane. Membrane estrogen, progestin and androgen receptor proteins have been cloned and identified. Genetic and pharmacologic studies demonstrate their function in the nervous system, skin, cardiovascular system and reproductive tract. Membrane estrogen, progestin and androgen receptor proteins respond to steroid ligands independently of the corresponding nuclear receptor. The existence of membrane glucocorticoid and mineralocorticoid receptors is less clear. While there is evidence of glucocorticoids and mineralocorticoids binding to cell membranes, the identity of membrane proteins to which they bind is unknown (Figure 1).

To make matters more confusing, nuclear hormone receptors can sometimes function at the plasma membrane. For example, nuclear estrogen receptors can be post-translationally modified by the covalent attachment of palmitic acid. Such palmitoylation anchors nuclear estrogen receptors to cell membranes and allows nuclear estrogen receptors to respond to steroids at the plasma membrane, independent of their function as transcription factors (1–7). In contrast to the nuclear receptors, integral membrane steroid receptors are transmembrane proteins in cell membranes. Integral membrane steroid receptors respond to steroids by activating or inhibiting other proteins and are not capable of directly regulating transcription. Nuclear receptors can bind DNA in the nucleus or activate second messenger cascades at the plasma membrane, whereas integral membrane steroid receptors can only signal at cell membranes. Here, we focus on integral membrane steroid receptors because their pharmacology and function is less well understood compared to nuclear steroid receptors.
Terminology in the field is confusing. Nuclear receptors, such as the progesterone receptor, are referred to as PR. Does membrane progesterone receptor (mPR) refer to a nuclear progesterone receptor (PR) that is anchored to the plasma membrane? Or does mPR refer to a progestin and adipoQ receptor (PAQR), integral membrane proteins that bind progestins at the plasma membrane? Or does mPR refer to PGRMC1, a single pass transmembrane protein that binds progestins? For clarity we use specific names (e.g., PAQR7 rather than mPRα).

What does it take to call an integral membrane protein a steroid receptor? Ideally, the membrane protein would be purified, reconstituted into lipid bilayers, and tested for specific binding. Purifying and reconstituting integral membrane proteins such that they maintain their activity in vitro is difficult and has been done for only two of the known or putative membrane steroid receptors (NavMs and TRPM8). The best alternative is comparing steroid binding to cell membranes in cells that do or do not express the putative membrane steroid receptor. Demonstrating steroid- and membrane steroid receptor-dependent activation of second messenger cascades, such as ERK phosphorylation, or changes in intracellular calcium are good supportive evidence. In vivo, there should be steroid- and protein-dependent phenotypes: expose wild-type animals to a steroid and measure a phenotype, expose animals with a mutation in the putative membrane steroid receptor to a steroid and the phenotype should be reduced or absent. In this review, we discuss to what extent the known or putative membrane steroid receptors meet these criteria.

What does it take to demonstrate that a membrane steroid receptor acts independently of its corresponding nuclear hormone receptor? As we discuss below, there are many cases where membrane receptor activity was detected in the absence of nuclear receptors, both in vitro
The most parsimonious assumption is that membrane steroid receptors act independently of nuclear steroid receptors, even though they may share a ligand.

I. Membrane estrogen receptors

G protein-coupled estrogen receptor

In 2005, the labs of Peter Thomas and Eric Prossnitz independently discovered that an orphan G protein-coupled receptor, GPR30, responds to estrogens (8,9). GRP30 is now known as the G protein-coupled estrogen receptor protein (GPER, encoded by the GPER1 gene). Competitive ligand binding assays to cells or cell membranes in cell lines that express GPER endogenously and in cell lines that overexpress transfected GPER demonstrated specific binding of estrogens to GPER. These studies also demonstrated lack of specific binding of progesterone, testosterone or cortisol. Estradiol also activated GPER-dependent second messenger pathways (8,9).

How do we know that GPER responds to estrogens independently of the nuclear estrogen receptors? The identification of synthetic small molecules that have differential activity towards GPER versus nuclear estrogen receptors alpha and beta allows scientists to selectively activate membrane or nuclear estrogen receptors in vivo and explore their functions independently (10–13).

Estradiol increased heart rate in zebrafish embryos via GPER (14). The fact that this effect was mimicked by exposure to a GPER-selective agonist, was blocked by a GPER-selective
antagonist, and that estradiol increased heart rate in nuclear estrogen receptor mutants but failed to increase heart rate in gper1 mutants, demonstrates that estradiol increases heart rate via GPER, independently of nuclear estrogen receptor proteins. Similarly, GPER activation increased melanin synthesis in mice and in cultured human melanocytes that do not express nuclear estrogen receptors (15). These studies demonstrate that GPER acts as an estrogen receptor in vivo independently of nuclear estrogen receptors alpha and beta.

Using selective GPER agonists and antagonists, together with animal models with targeted mutations in GPER, scientists discovered that GPER influences the development and function of many tissues and cell types. We highlight a few examples here, for more details see (16).

GPER activity protects the fetus from maternal inflammation in mice (17). GPER promotes liver growth in zebrafish embryos and proliferation of cultured human primary hepatocytes (18). GPER activation causes vasodilation in carotid arteries and reduces mean arterial pressure in mice and rats (19,20). In three different species of fish, GPER activity mediates oocyte maturation by blocking maturation and maintaining meiotic arrest (21–24). In contrast, progestins promote oocyte maturation via membrane progesterone receptors (more on that below). GPER regulates energy balance in post pubertal mice (25) and stimulates acute calcium signaling and H+-ATPase activity in renal tubules and intercalated cells (26). In estrogen-induced thymic atrophy, GPER promotes thymocyte apoptosis (27).

GPER is also a therapeutic target for the treatment of different diseases. GPER agonists are being developed to treat melanoma (28), obesity, diabetes and metabolic disorder (29), and multiple sclerosis (30–33). GPER antagonists could help treat hepatocellular carcinoma (18).
Voltage-gated sodium channel Nav1.2

Tamoxifen, a selective estrogen receptor modulator, can bind a sodium channel from *Magnetococcus marinus* (NavMs) and inhibit NavMs and the human homolog Nav1.2 *in vitro* (34). Scientists often ignore the unintended targets of estrogens. We argue that such off-target proteins should be recognized as membrane estrogen receptors. NavMs is one of the few examples of a crystal structure demonstrating an estrogen receptor ligand directly bound to an ion channel. There is additional indirect evidence for estrogens activating or inhibiting ion channel activity (reviewed in (35)). Some ion channels, despite their name, may also be membrane estrogen receptors.

II. Membrane progestin receptors

Non-nuclear actions of progestins have been recognized for years and have often been attributed to extra-nuclear or membrane-associated activities of the classical nuclear progesterone receptor (PR). However, several studies demonstrate a role for progestin signaling in cells and tissues that lack classical PR expression, suggesting that alternative, membrane-localized progestin receptors exist. In fact, membrane progestin receptors have been identified and they belong to two different families of receptors: the progestin and adipoQ (PAQR) family and the B5-like heme/steroid binding protein family.
PAQR7 (mPRα)

The PAQR family consists of 11 receptors in humans, with 5 that function as membrane progestin receptors (PAQR5-9) (36). The best characterized member of this family is PAQR7 (also known as mPRα). PAQR7 was initially discovered in teleost fish (37), and was subsequently identified in humans (38) and in other mammalian species (reviewed in (39)). Ligand binding to PAQR7 was demonstrated via [3H]progesterone binding 1) to recombinant receptor (38), 2) to plasma membrane fractions from cells transfected with PAQR7 (40–42) or from cells with endogenous PAQR7 expression (43), and 3) in whole BxPC3 pancreatic adenocarcinoma cells that express high levels of PAQR7 (44). Another study utilized P4 conjugated to BSA and FITC to demonstrate the presence of progestin binding sites on the surface of myometrial cells (45). PAQRs have been shown to activate a second messenger signaling pathway culminating in repression of the FET3 gene in transfected yeast (46). This same assay was used to demonstrate that PAQR7 responds to progesterone at physiologically relevant concentrations (47). This effect was specific, as ligand-dependent repression of FET3 was not seen in cells transfected with empty vector or with human adiponectin receptors, nor in cells treated with cortisol or testosterone. It should be noted that one study did not report PAQR7 binding to progesterone to mediate nongenomic actions (48). In this study, however, the transfected PAQR7 were localized to the endoplasmic reticulum and not to the plasma membrane.

Researchers have been hunting for selective ligands that bind with high affinity to PAQR7, but with low or no affinity to nuclear PR (41,42,44,49). Only one identified PAQR7 ligand, Org OD 02-o, was used to interrogate the physiological function of PAQR7 in vivo. In fish, administration of Org OD 02-o is associated with oocyte maturation (50) and sperm motility (51–53). Administration of Org OD 02-o was associated with decreased prolactin serum levels in a mouse model of prolactinoma and with decreased prolactin release in pituitary explants (54). Studies utilized Org OD 02-o in vitro to demonstrate that PAQR7 activity
inhibits melanin production (15) and mediates vascular smooth muscle relaxation (55,56). A major caveat to these studies is that while Org OD 02-0 has 20-fold higher affinity for PAQR7 compared to nuclear PR (49), it can still bind nuclear PR, so a role for the classical receptor in the observed effects cannot entirely be ruled out. A more direct approach using antisense oligonucleotides targeted against PAQR genes in the rat midbrain demonstrated a role for membrane progestin receptors in progesterone-facilitated lordosis (57,58). Finally, knockdown of PAQR7 expression, but not nuclear PR, decreased progesterone-mediated GLP-1 secretion in enteroendocrine cells in vitro (59).

PAQRs may also contribute to cancer development, proliferation, and metastasis. The majority of the supporting data for this role comes from in vitro/xenograft studies or analyses of receptor expression in tumors (39,60). In one xenograft study, knockdown of PAQR7 impaired the inhibitory effects of progesterone on tumor growth and metastasis of triple-negative breast cancer cells (61). However, it should be noted that conflicting data exist regarding PAQR7 overexpression in specific breast cancer subtypes (61,62). In another xenograft study, administration of Org OD 02-0 inhibited the growth of lung adenocarcinoma cells (63). Interestingly, PAQR7 expression is associated with epidermal growth factor receptor (EGFR) expression/mutations in breast and lung tumors (62,64), supporting the hypothesis that PAQR7 is a possible biomarker for tumors that may benefit from targeted EGFR therapies. PAQR7 expression was associated with lymph node metastasis in breast cancer patients (65) and poor prognosis in lung cancer patients (63), highlighting the need to better understand the exact role of PAQR7 in tumorigenesis.
PAQR8 and PAQR5 (mPRβ, mPRγ)

PAQR8 (mPRβ) and PAQR5 (mPRγ) were cloned and discovered at the same time as PAQR7 (38). However, much less has been reported about the ligand binding properties, the tissue selectivity, and the physiological function of these two receptors compared to PAQR7. A reporter gene assay in transfected yeast has demonstrated that both PAQR8 and PAQR5 respond to progesterone at physiologically relevant concentrations (47). Ligand-binding to PAQR8 was also demonstrated by [3H]progesterone binding to plasma membrane fractions of transfected ovarian cells (66). As for physiological function, one study demonstrated that PAQR8 promotes progesterone-dependent oocyte maturation in Xenopus (66). Another study demonstrated that inhibition of PAQR8 expression in the brain stem via siRNA reduced sleep apnea frequency in mice (67). However, scramble siRNA controls were not used in these experiments, making it difficult to rule out possible off-target effects of siRNA administration. Interestingly, decreased expression of PAQR8 was observed in endometrial tumors with lymphovascular invasion and decreased PAQR5 expression was observed with increasing FIGO stage of endometrial tumors, suggesting that both receptors may be prognostic markers in endometrial cancer (68). Similar to what was observed with PAQR7, knockdown of PAQR5, but not nuclear PR, expression decreased progesterone-mediated GLP-1 secretion in enteroendocrine cells (59). It is clear that future studies using knockdown of expression, selective ligands, and/or mutations in model organisms are needed to further determine the functional significance of these two receptors.
PAQR6 (mPR\(\delta\)) and PAQR9 (mPR\(\varepsilon\))

Phylogenetic analyses of the PAQRs suggested that there were two additional members of this family, PAQR6 and PAQR9 (40). These receptors were subsequently named mPR\(\delta\) and mPR\(\varepsilon\) (47). Both receptors bind progesterone and PAQR7 agonists (and to a lesser extent testosterone, but not cortisol or estradiol) in the transfected yeast reporter gene assay discussed above (47) and by \(^{3}H\)progesterone binding to plasma membrane fractions from transfected breast cancer cells (69). Treatment of transfected breast cancer cells with progesterone or PAQR7 agonists activated a stimulatory G protein, in contrast to PAQR7 and PAQR8 that activate an inhibitory G protein (69). While PAQR9 is expressed in a variety of tissues, PAQR6 expression is limited to the brain (36,69). Interestingly, PAQR6 was shown to have the highest affinity for neurosteroids compared to PAQR7, PAQR8, and PAQR9 (69), suggesting that PAQR6 may play an important role in mediating neurosteroid, particularly neuroprotective, actions in the brain. However, the functions of PAQR6 and PAQR9 \textit{in vivo} remain poorly understood.

PGRMC1

There are 4 members of the B5-like heme/steroid binding protein family: progesterone membrane component 1 (PGRMC1; also known as IZA, sigma- receptor, Dap1), PGRMC2, neudesin (NENF), and neufercin (CYB5D2). They share a similar non-covalent heme-binding domain that is related to cytochrome b5. PGRMC2, neudesin and neufercin have not been shown to bind progestins (70–72).
PGRMC1 was originally discovered from porcine and rat liver membranes independently by two groups in 1996 (73) and was subsequently cloned in 1998 (72). Historically, PGRMC1 has been known as 25-Dx, membrane PR or mPR, Hpr6, VemaA, Ratp28, and IZA (reviewed in (74)). A comprehensive discussion of this history is beyond the scope of this review but was mentioned here to raise awareness of alternative synonyms in the literature and to highlight the multifaceted functions attributed to PGRMC1. Evidence for progesterone/steroid binding to PGRMC1 is based on ligand binding to microsomal membranes containing PGRMC1 (and presumably other proteins) or to cells overexpressing PGRMC1 (73,75–77), and on direct progestin binding via spectroscopic analysis (78). Deletion mutations of PGRMC1 in granulosa cells suggest that the entire molecule is required for maximal progestin binding and responsiveness (79). It should be noted that PGRMC1 can also bind heme and cholesterol (74,80,81), which is consistent with its reported functions in different cellular contexts.

The physiological relevance of PGRMC1 in vivo has been explored via knockdown of receptor expression. For example, conditional knockdown of Pgrmc1 in the female reproductive tract resulted in subfertility and development of endometrial cysts in mice (82). Global knockout of pgrmc1 in zebrafish resulted in reduced fertility, presumably due to impaired oocyte maturation (83). Similar results were obtained in a follow-up study, where double knockout of pgrmc1/2 resulted in reduced fertility, presumably due to reduced oocyte ovulation (84). A role for PGRMC1 in granulosa cell mitosis and survival was also demonstrated via conditional knockout in murine granulosa cells (85). Global knockout of PGRMC1 impaired mammary gland development in mice (86). Conditional double knockout of PGRMC1 and PGRMC2 in luteal cells of the mouse ovary resulted in decreased corpus luteum vascularization and decreased primary follicles in adulthood (87). Together, these studies support a role for PGRMC1 in reproductive health and disease. PGRMC1 may also play a role in metabolic health and disease. For example, global knockout mice fed a high fat diet
exhibited increased levels of hepatic triglycerides and were predisposed to non-alcoholic fatty liver disease when compared to wild-type mice (88). In addition, adipose-specific knockout of PGRMC1 reduced adipocyte hypertrophy in mice fed a high-fat diet, suggesting a role for this receptor in the development of obesity (89).

Interestingly, PGRMC1 has also been shown to be overexpressed in a variety of tumors, including lung, thyroid, colon, ovarian, cervical, and breast (reviewed in (90)). Knockdown of PGRMC1 in xenograft models of colon (91), breast (92), endometrial (93), and ovarian (94) tumorigenesis led to reduced tumor growth and/or metastasis. In xenograft models of breast cancer, overexpression of PGRMC1 resulted in increased breast tumor growth, with (95–97) or without (98) hormone treatment. The studies with hormone treatment provide support for the hypothesis that PGRMC1 may be associated with the increased risk of breast cancer associated with hormonal therapy (reviewed in (90)). Treatment with progestin was shown to stimulate phosphorylation of PGRMC1 at Ser181 (a CK2 protein kinase phosphorylation site) in breast cancer cells overexpressing this receptor, suggesting one possible mechanism of progestin-mediated activation of PGRMC1 in cancer cells (99). It should be noted that high expression levels of PGRMC1 are associated with worse response to neoadjuvant therapy (100) and with aggressive phenotype and poor prognosis (101) in breast cancer patients, highlighting the need to better understand the role of PGRMC1 in tumorigenesis. Although in vitro studies have examined the mechanistic effects of PGRMC1 action on hallmarks of cancer, those studies that utilized the PGRMC1 inhibitor AG-205 should be interpreted with caution as the specificity of this inhibitor has not been thoroughly established (reviewed in (102)).
III. Membrane androgen receptors

There is evidence for androgens binding membrane proteins \textit{in vitro}. However, the evidence for androgen-dependent function of such proteins \textit{in vivo} is less well established, especially compared to membrane estrogen & progestin receptors. For detailed review of evidence for androgen binding membrane receptors \textit{in vitro}, see (103).

ZIP9

Zrt- and Irt-like protein 9 (ZIP9, encoded by the \textit{SLC39A9} gene) is a member of the ZIP family of zinc transporter proteins. Competitive ligand binding assays to cell membranes using radioactive testosterone, in cultured cells that express human ZIP9 endogenously and in cells that overexpress transfected ZIP9, demonstrated specific binding (104,105). In terms of second messenger pathways, testosterone-dependent ZIP9 activity increased G protein activation, expression of Bax, p53 and JNK proteins, and activated Erk1/2, CREB and ATF-1 proteins in various cell lines from Atlantic croaker fish, rats and mice (104–108). In human and mouse melanoma cells, testosterone increased intracellular zinc concentrations in a ZIP9-dependent manner ((109), note that this a preprint and has not been peer-reviewed). Thus far there is little evidence that androgen-dependent ZIP9 activity in cultured cells requires nuclear androgen receptors.

There are no known steroids or steroid-like compounds that have well-characterized differential affinity for ZIP9 versus nuclear androgen receptors. Testosterone and dihydrotestosterone activate nuclear androgen receptors and ZIP9, although ZIP9 has higher affinity for testosterone compared to dihydrotestosterone (105). Cyproterone acetate blocks
nuclear androgen receptor activity but did not reduce testosterone and Zip9-dependent proliferation in melanoma cells (109), suggesting that cyproterone acetate has higher affinity for nuclear androgen receptors than for ZIP9. In contrast, nuclear androgen receptor antagonists such as bicalutamide, enzalutamide and apalutamide appear to similarly antagonize ZIP9 (109).

ZIP9 may act in vivo in both an androgen-dependent and androgen-independent manner. Distinguishing between the two has been challenging. Female zebrafish zip9 mutants exhibited reduced fecundity and egg viability compared to wild type. Additionally, oocytes from zip9 mutants exhibited reduced diameter cortical vesicles and smaller zinc-containing vesicles compared to oocytes from wild-type fish (110). These results suggest that zinc influences egg activation, but they do not address whether intracellular zinc concentration or ZIP9 function in oocytes is androgen dependent. ZIP9 could be acting in an androgen-independent manner. Future studies are required to carefully determine when and how ZIP9 acts as an androgen receptor in vivo.

**GPRC6A**

G protein-coupled receptor family C group 6 member A (GPRC6A) is a GPCR activated by calcium, magnesium and the peptide hormone osteocalcin. In cultured cells transfected with GPRC6A, radioactive and membrane-impermeable testosterone bound cell membranes compared to cell membranes from non-transfected cells (111). There is also evidence that androgens activate second messenger signaling via GPRC6A. Membrane impermeable testosterone and the synthetic androgen R1881 increased levels of phosphorylated ERK in cultured cells transfected with GPRC6A compared to non-transfected cells. Androgen-dependent ERK activation was blocked by a G protein inhibitor, consistent with the
hypothesis that androgens activate GPRC6A (111,112). In cultured keratinocytes, testosterone treatment increased intracellular calcium and H2O2. These effects were reversed by knockdown of GPRC6A (113). In bone marrow stromal cells cultured from wild-type or GPRC6A mutant mice, cells from wild-type mice exposed to testosterone had increased phosphorylated ERK compared to cells from mutant mice (111). In wild-type mice, testosterone treatment increased levels of phosphorylated ERK and Egr-1 expression in bone marrow and testis. These responses were absent or diminished in GPRC6A mutant mice (111). These studies suggest that GPRC6A acts as an androgen-receptor in vivo.

It is not known whether nuclear androgen receptor antagonists block GPRC6A activity. There are no known steroids or steroid-like compounds that have high affinity for GPRC6A but not nuclear androgen receptors, or vice versa.

**OXER1**

Oxoeicosanoid receptor 1 (OXER1) is a GPCR activated by arachidonic acid metabolites such as 5-oxoeicosatetraenoic acid (5-oxo-EXE). In cultured prostate cell lines, labeled testosterone bound to plasma membrane, and binding was reduced by exogenous 5-oxo-EXE or by knocking down endogenous OXER1 (114). Similar results were obtained using Chinese Hamster Ovary cells overexpressing OXER1. 5-oxo-EXE inhibits cAMP production via OXER1 and Gai, these effects were reversed by addition of testosterone (114). Together, these results suggest that testosterone binds OXER1 and blocks activity. Whether OXER1 responds to androgens in vivo is not known. In cultured adrenocortical cells, activation of OXER1 by 5-oxo-EXE increased steroidogenesis (115,116), but whether this activity of OXER1 is influenced or blocked by androgens is not known.
TRPM8

Transient receptor potential cation channel subfamily M member 8 (TRPM8) is a nonselective cation channel (sodium, calcium) that is activated by cold temperatures and cooling agents such as menthol (117,118). In contrast to most of the membrane steroid receptors, TRPM8 has been purified and shown to bind testosterone in vitro. Binding activated ion channel activity, which was blocked by TRPM8 antagonists (119). Additionally, testosterone specifically bound to cell membranes that endogenously or exogenously express TRPM8 (119,120).

The function of TRPM8 as a cold sensor has been characterized in vivo, but androgen-dependent functions of TRPM8 in vivo are not clear. Mohandass and colleagues used TRPM8 mutant mice to explore whether TRPM8 plays a role in androgen-dependent behaviors, such as mating and aggression. Compared to controls, TRPM8−/− male mice displayed a markedly increased frequency in mounting TRPM8−/− females, while TRPM8−/− males exhibited more aggression toward their conspecifics (121). This change in androgen-dependent behavior correlates with the absence of TRPM8, an androgen-receptor in vitro. But correlation does not equal causation. Mating and aggression are complex behaviors regulated by many stimuli, not just androgens. TRPM8 is an ion channel with multiple ligands, not just testosterone. It is possible that TRPM8 influences mating and aggression behaviors independently of binding to androgens. Further studies are required to determine the effects of androgen-dependent activation of TRPM8 in vivo.
The alpha 1 subunit of the L-type voltage-dependent calcium channel (Cav1.2), encoded by the CACNA1C gene, forms the pore through which divalent ions traverse cell membranes. Mutations in CACNA1C are associated with abnormal cardiac conduction: Timothy Syndrome, Brugada Syndrome, and Long QT Syndrome 8 (122–128).

Using whole-cell patch-clamp recordings, Chris Peers and colleagues demonstrated that testosterone inhibited Cav1.2 currents in stably transfected cells and in a rat aortic smooth muscle line that expresses Cav1.2 endogenously. This effect was specific for testosterone because 5α-androstan-17β-ol-3-one, progesterone and estradiol had no effect (129,130).

Mouse and zebrafish with mutations in Cav1.2 exhibit cardiac phenotypes (131–134), but whether these phenotypes are influenced by androgens binding to Cav1.2 is not known.

IV. Membrane glucocorticoid receptors

Evidence for the existence of membrane-associated glucocorticoid receptor (here referred to as mGR) comes from studies that isolated plasma membrane fractions and demonstrated ligand (cortisol/corticosterone or dexamethasone) binding to these fractions (135–146). Immunofluorescence microscopy studies showed nuclear GR localization to the plasma membrane in liver (142), in brain (147), in immune cells (148–150) and in leukemia/lymphoma cell lines (151,152). Despite this evidence, the molecular structure of a
unique mGR has yet to be characterized. It is possible that nuclear GR and mGR are encoded by the same gene (153, 154).

Studies have suggested that nuclear GR associates with the plasma membrane through interaction with the scaffolding protein caveolin-1. For example, nuclear GR was localized to caveolin-containing membrane fractions in human lung cancer cells, and this interaction was necessary for glucocorticoid-mediated activation of second messenger pathways (155). Proximity ligation assays also demonstrated the physical association of caveolin-1 and GR in U2-OS and MCF-7 cells (154). However, this interaction may be cell- and context-dependent as CAV1 and GR were not colocalized in human monocytes (149). Although GR does have a palmitoylation sequence, neither mutations of this site nor a palmitoylation inhibitor prevented membrane localization of GR in transfected COS-7 cells (156). Furthermore, GR was not shown to undergo palmitoylation and colocalized with caveolin-1 in these cells (156). This is in contrast to what has been observed for ER, where mutation of the palmitoylation site prevented interaction with CAV1 and membrane localization (3, 7, 157). Membrane localization of PR and AR is also inhibited by mutation of the palmitoylation site or by knockdown of palmitoylacyltransferase proteins (6, 7). These results suggest that the subpopulations of palmitoylated and of caveolin-associated ER, PR, and AR, but possibly not GR, are one and the same.

The physiological function of mGR in vivo has been interrogated by using bovine serum albumin (BSA)-conjugated glucocorticoids that are membrane impermeable. For example, treatment with cortisol-BSA mediated the stress response in skeletal muscle of fish (158–160). In addition, treatment with cortisol-BSA altered glucose metabolism in fish (161). While these studies indirectly support a role for mGR, note that the use of BSA-conjugated glucocorticoids does not rule out a role for the transcription factor activity of nuclear GR. For
example, BSA-glucocorticoid induced expression of a GR-responsive reporter gene (162). In addition, treatment with BSA-dexamethasone for 30 minutes increased translocation of nuclear GR from the cytoplasm to the nucleus in hypothalamic neurons in the rat, suggesting either that membrane signaling can regulate the transcriptional activity of nuclear GR or that BSA-dexamethasone may in fact be membrane permeable (163). In light of the fact that glucocorticoids are used as therapeutics for a variety of human diseases, deciphering the specific roles of nuclear GR and mGR in target tissues warrants further study.

V. Membrane mineralocorticoid receptors

To date, the protein(s) by which mineralocorticoids act at the cell membrane has not been identified. However, mineralocorticoid binding to plasma membranes containing nuclear mineralocorticoid receptor (MR) from mammalian cells has been demonstrated (164–167). Furthermore, MR was detected at the plasma membrane in mammalian kidney (168) via microscopy. Unlike other steroid receptors, MR lacks a palmitoylation site, therefore it does not seem to be directly anchored to the membrane. There is evidence that MR is associated with the cytosolic side of the plasma membrane via interaction with scaffolding proteins, such as striatin and caveolin-1 (reviewed in (169)). For example, aldosterone rapidly activated ERK signaling in mouse and human endothelial cells in a striatin-dependent manner as reduction of striatin levels with siRNA prevented this activation (170). Importantly, caveolin was shown to be necessary for the interaction of MR with striatin, as there was no discernable interaction in tissues from caveolin knockout mice (170). In mice fed a high sodium diet, caveolin-1 and MR expression, as well as interactions between the two, were increased (171). Striatin has been shown to affect physiological responses dependent on aldosterone/MR activation, such as salt-sensitive blood pressure and renal
damage (172–174). Caveolin-1 has been shown to modulate glucose and lipid homeostasis, which are also aldosterone-associated physiological responses (175).

There is also evidence that MR interacts with membrane receptors such as epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR), insulin-like growth factor 1 (IGF1R), angiotensin receptor (AT1), and GPER1 (reviewed in (169)). For example, MR was shown to colocalize with EGFR at the plasma membrane in kidney cells (168), and EGFR has been shown to be a mediator of aldosterone-induced reabsorption of sodium in kidney cells (reviewed in (176)). Membrane-associated MR signaling may involve crosstalk with multiple membrane receptors. In breast cancer and breast tumor-associated endothelial cells, aldosterone-mediated activation of EGFR was blocked by GPER1 knockdown and by treatment with the GPER1 antagonist G15 (177). Furthermore, aldosterone treatment increased the interaction of MR and GPER1 and was associated with proliferation/migration of these cells (177). It should be noted that aldosterone can have effects in cells that do not express MR, prompting the hypothesis that another receptor may be responsible for these effects. Currently, there is debate as to whether GPER1 is the receptor responsible for membrane-associated aldosterone action even though direct binding of aldosterone to GPER has not been shown (178–180). This is an interesting concept, as GPER1 is potentially druggable and hence relevant from a translational perspective. More studies are needed to definitively identify the membrane form of MR and determine its physiological relevance.

**Conclusions and future directions**

Bona fide integral membrane estrogen, progestin and androgen receptors exist, distinct from their nuclear receptor counterparts. However, we have much to learn about their structure and function. Most integral membrane steroid receptors have not been purified for ligand binding assays. We have no high-resolution 3D structures for these receptors. Additionally,
we lack high-throughput assays to measure ligand binding, which would be useful to identify and characterize environmentally relevant ligands, such as endocrine disrupting compounds, that bind integral membrane steroid receptors. High-throughput assays to measure G protein-coupled receptor activity, such as TANGO (181), have not yet been used to assay GPER activity.

One feature of nuclear hormone receptors is that they are modular in structure. Each nuclear receptor contains a ligand binding domain that is similar in sequence and structure among all nuclear receptors. It is not clear whether membrane steroid receptors have ligand binding domains with a high degree of sequence and structural similarity to each other.

The significance of membrane steroid receptors in health and disease is underappreciated. Accumulating evidence suggests that membrane steroid receptors may play a role in cancer development and/or progression. The best evidence of this exists for the PAQR membrane progestin receptors. More work is needed to elucidate the role of other membrane steroid receptors in cancer. Steroid hormone signaling can be targeted for therapeutic purposes in a variety of diseases. For example, antagonists to nuclear estrogen and androgen receptors are used to treat different cancers. Unfortunately, many of these treatments are associated with unwanted side effects. In light of the fact that estrogens, androgens and progestins can activate nuclear and membrane signaling pathways, it is intriguing to speculate that steroid receptor antagonists that are selective for membrane versus nuclear receptors (or vice versa) may, in some situations, be more suitable therapeutics with fewer side-effects.
Figure 1. Steroid hormone receptors at the cell membrane. Integral membrane proteins, such as G protein-coupled receptors (GPCR), ion channels, and transporters can bind steroid hormones estrogens, androgens and progestins as shown here. Additionally, soluble nuclear hormone receptors, such as estrogen receptor alpha and glucocorticoid receptor, can be anchored to the plasma membrane via post-translational modification and/or association with caveolin.

Data Availability: Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.
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