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Characteristics of membrane progesterin receptor alpha (mPR α) and progesterone membrane receptor component one (PGMRC1) and their roles in mediating rapid progesterin actions

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Abstract

Rapid, progesterin actions initiated at the cell surface that are often nongenomic have been described in a variety of reproductive tissues, but until recently the identities of the membrane receptors mediating these nonclassical progesterins actions remained unclear. Evidence has been obtained in the last 4-5 years for the involvement of two types of novel membrane proteins unrelated to nuclear steroid receptors, progesterone membrane receptors (mPRs) and progesterone receptor membrane component one (PGMRC1 [also known as progesterone receptor membrane component 1, PGRMC1]), in progesterin signaling in several vertebrate reproductive tissues and in the brain. The mPRs, (MW ~40 kDa) initially discovered in fish ovaries, comprise at least three subtypes, α , β and γ and belong to the seven-transmembrane progesterone adiponectin Q receptor (PAQR) family. Both recombinant and wild type mPRs display high affinity (K_d ~5 nM), limited capacity, displaceable and specific progesterone binding. The mPRs are directly coupled to G proteins and typically activate pertussis-sensitive inhibitory G proteins (G_i), to down regulate adenylyl cyclase activity. Recent studies suggest the alpha subtype (mPR α) has important physiological functions in variety of reproductive tissues. The mPR α is an intermediary in progesterin induction of oocyte maturation and stimulation of sperm hypermotility in fish. In mammals, the mPR α s have been implicated in progesterone regulation of uterine function in humans and GnRH secretion in rodents. The single-transmembrane protein PGMRC1 (MW 26 ~28 kDa) was first purified from porcine livers and its cDNA was subsequently cloned from porcine smooth muscle cells and a variety of other tissues by different investigators. PGMRC1 and the closely related PGMRC2 belong to the membrane-associated progesterone receptor (MAPR) family. The PGMRC1 protein displays moderately high binding affinity for progesterone which is 2-10-fold greater than that for testosterone and glucocorticoids, and also can bind to other molecules such as heme, cholesterol metabolites and proteins. The signal transduction pathways induced by binding of progesterone to PGMRC1 have not been described to date, although motifs for tyrosine kinase, kinase binding, SH2 and SH3 have been predicted from the amino acid sequence. Evidence has been obtained that PGMRC1 mediates the antiapoptotic affects of progesterone in rat granulosa cells. The PGMRC1 protein may also be an intermediary in the progesterone induction of the acrosome reaction in mammalian sperm. Despite these recent advances, many aspects of progesterin signaling through these two families of novel membrane proteins remain unresolved. Biochemical characterization of the receptors has been hampered by rapid degradation of the partially purified proteins. A major technical challenge has been to express sufficient amounts of the recombinant receptors on the plasma membranes in eukaryotic systems to permit investigations of their progesterin binding and signal transduction characteristics. Additional basic information on the molecular and cellular mechanisms by which

mPRs and PGMRC1 interact with progestins, signal transductions pathways and other proteins will be required to establish a comprehensive model of nontraditional progestin actions mediated through these novel proteins.

Introduction

Many effects of steroids cannot be readily explained by the classic genomic mechanism of steroid action involving new mRNA and protein synthesis which is relatively slow, typically occurring over a time scale of hours to days. Steroids have been shown to initiate rapid actions through activation of intracellular signaling pathways, resulting in alterations in ion fluxes and intracellular free calcium concentrations occurring within seconds [6], and of other second messengers such as cyclic nucleotides and extracellular-regulated kinase 1 and 2 (erk 1/2), within a few minutes [30,150]. The majority of studies have shown that these rapid steroid actions are initiated at or near the cell surface and evidence has accumulated that they are mediated by binding to specific receptors in the plasma membranes of the target cells [28,75, 102,103,118,142]. However, steroids readily diffuse into cells where they can also activate intracellular steroid receptors to rapidly initiate signal transduction pathways [10,111]. The biological response mediated through steroid membrane receptors can be rapid, such as the stimulation of sperm hypermotility within 1-5 minutes of progestin treatment, or can occur over a prolonged period of 6-18hrs in the case of oocyte maturation in fish and amphibians after treatment with the same steroids [118,121]. Progestins induction of oocyte maturation through progestin membrane receptors is via a nongenomic mechanism since it is not blocked by inhibitors of transcription and translation [20,128]. However, progestin binding to progestin membrane receptors in oocytes also activates MAPkinases [82] which is likely to ultimately result in alterations in gene transcription. In addition, progestin membrane receptor-mediated pathways can also regulate transactivation of the nuclear progesterone receptor resulting in alteration in gene transcription [41]. Thus the only characteristic common to all these nonclassical steroid actions is rapid activation of intracellular signaling pathways.

The receptors mediating these rapid steroid actions have been studied extensively in many laboratories over the past 20 years. For example binding moieties with the characteristics of progestin membrane receptors have been demonstrated in fish and amphibian oocytes [55, 84] in bovine ovaries [97,98]), in rat Leydig cells [106] in human and fish sperm membranes [6,53,26,120,127], in pig liver microsomes [63] and for progesterone and progesterone metabolites in breast cancer cells [23,144]. However, the identities of the receptors mediating many nonclassical actions of progestins and other steroids, their precise locations in the cell and their mechanisms of action remain unclear and are surrounded by controversy. A variety of receptor proteins have been proposed as intermediaries in nonclassical steroid actions in various cell models. Candidates for these receptors include nuclear steroid receptors or steroid nuclear receptor-like forms that are membrane-bound [101,143] or can be activated intracellularly [66], receptors for other ligands that also bind steroids [70,72,105], novel receptor proteins unrelated to any previously described receptors [104,123], and receptors with different characteristics from those of any known receptors [49,63]. For example, a diverse range of receptor proteins have been implicated in nonclassical progestin actions in different vertebrate and cell models, including nuclear receptors that can activate intracellular signaling cascades [3,10,111,130] GABA_A and oxytocin receptors that can bind progesterone [32,34] and two novel receptor families which are unrelated to any previously described receptor proteins, membrane progestin receptors (mPRs) and progesterone membrane receptor component 1 [27,150].

The identification of the genes encoding mPRs and PGMRC1 and a knowledge of their protein structures has generated broad interest amongst researchers resulting in recent advances in our

understanding of their likely physiological functions. The characteristics of these two novel putative progestin membrane receptors and their roles in mediating rapid progestin actions in a variety of target tissues and cells are reviewed in this paper. Evidence that mPR α (mPR α) and PGMRC1 function as membrane progestin receptors is critically evaluated. In addition, methodological problems in progestin membrane receptor measurement and expression of recombinant receptors in plasma membranes are discussed. The involvement of nuclear progesterone receptors in mediating rapid progestin actions involving activation in second messengers will not be discussed because there is an excellent recent review on this topic [9].

Membrane progestin receptor alpha-mPR α

1. Progestin membrane receptors in fish and amphibian oocytes

Meiotic maturation of fish and amphibian oocytes is one the most extensively studied and well-characterized rapid, nonclassical progestin actions mediated by activation of membrane bound steroid receptors at both the biochemical and cytological levels [55,75,146]. The presence of progesterone binding sites on the cell surface of amphibian oocytes was demonstrated in the early 1980s, and specific progestin binding to the membrane receptors in *Xenopus* and *Rana* oocytes has been well characterized [8,45,50,71,109]. The *Xenopus* membrane receptor displays high affinity progesterone binding (K_d 10^{-9} M) [50], whereas lower progesterone binding affinity has been reported for the *Rana* receptor (K_d 10^{-7} M) [45]. The nuclear progestin receptor agonist, R5020, also binds to the *Xenopus* membrane receptor, but with much lower affinity than progesterone (10^{-5} - 10^{-6} M) [8,109], which suggests that these receptors are unrelated to nuclear receptors [50]. Association of [H^3]-progesterone binding to *Xenopus* plasma membranes is rapid, similar to that of other steroid membrane receptors, reaching equilibrium within 15 min [50]. Finally, oocyte receptor abundance changes during the ovarian cycle. Receptor concentrations in small oocytes (stage IV) incapable of undergoing maturation in response to progesterone are about one tenth of those in full grown stage V/VI oocytes which are responsive to the steroid [50]. Moreover, incubation of ovarian fragments with a concentration of gonadotropin that promotes oocyte maturation causes receptor concentrations to increase several-fold to reach peak levels at the beginning of oocyte maturation [50]. These changes in the receptor abundance on *Xenopus* oocytes are consistent with a role for the receptor as the intermediary in progesterone induction of oocyte maturation in this species.

Fish membrane progestin receptors were first characterized extensively in spotted seatrout ovaries [84]. Spotted seatrout ovarian membranes exhibit a single class of high affinity (K_d 5 nM), low capacity, saturable and displaceable binding sites for 17,20 β ,21-trihydroxy-4-pregnen-3-one (20 β -S), the progestin hormone in this species that initiates oocyte maturation (maturation inducing steroid, MIS). The binding is specific for progestins and 20 β -S, which has hydroxyls at the 17, 20 and 21 positions, has the highest binding affinity for the receptor. The relative binding affinities of progesterone and the other major teleost progestin hormone that stimulates oocyte maturation, 17, 20 β -dihydroxy-4-pregnen-3-one (17,20 β DHP), are 6% and 0.6%, respectively, of the affinity of 20 β -S [121]. There is a close correlation between the relative binding affinities of various progestins and 11-deoxycorticosteroids for the spotted seatrout progestin membrane receptor and their agonist or antagonist activities in an *in vitro* oocyte maturation bioassay, suggesting the receptor is the likely intermediary in 20 β -S induction of oocyte maturation in this species [121]. The rates of association and dissociation of 20 β -S binding to ovarian membranes are rapid ($t_{1/2}$ 2-5 min). The binding kinetics and steroid specificity of the membrane progestin receptor in spotted seatrout differs markedly from those of the nuclear progestin receptor that controls ovulation in this species [96], suggesting that the membrane receptor is a novel protein unrelated to nuclear receptors. Membrane progestin receptors have also been identified in ovaries and oocytes of striped bass, rainbow trout, arctic char, yellow tail and goldfish [5,44,99,132,149]. The membrane progestin

receptors of rainbow trout, arctic char and goldfish show higher binding affinities for 17,20 β DHP than for 20 β -S, which is consistent with its identity as the likely MIS in these species. Similar to the observations in amphibians, membrane progesterin receptor concentrations increase in fish ovaries during oocyte maturation. In spotted seatrout, ovarian receptor concentrations increase two- to three-fold *in vivo* during oocyte maturation in fish collected on their spawning grounds which is accompanied by elevated plasma levels of LH and 20 β -S and also in captive fish induced to undergo oocyte maturation by LHRHa injection [125]. Tissue culture experiments show that receptor concentrations are also upregulated more than three-fold *in vitro* in ovarian fragments containing fully grown seatrout ovarian follicles incubated with gonadotropin which is associated with the development of oocyte maturation competence [85,125]. Progesterin membrane receptor concentrations are also increased in rainbow trout, arctic char and yellow tail ovaries after gonadotropin treatment [5,99,149]. This increase in oocyte receptor concentrations is physiologically important because it is associated with the oocytes becoming responsive to the MIS (oocyte maturational competence) and being able to complete oocyte maturation.

2. Cloning, identification and characterization of spotted seatrout membrane progesterin receptor alpha (mPR α)

Until recently, despite intensive research in several laboratories, efforts to characterize MIS progesterin receptors in lower vertebrates at the molecular level had been unsuccessful [56]. Several features of the seatrout receptor made it an attractive candidate for membrane receptor structure identification. The results from the binding studies suggested that the receptor protein is unrelated to the nuclear progesterone receptor and may be the product of a novel gene. In addition, the seatrout ovary is an abundant source of material for protein purification, since it comprises as much as 15% of the body weight and can contain up to half a million ovarian follicles with high levels of receptor expression undergoing oocyte maturation. Finally, extensive information was available of the biochemical characteristics of the receptor, its fluctuations during the reproductive cycle and its hormonal regulation. Most previous attempts to obtain highly purified preparations of steroid membrane receptors for amino acid sequencing using classical protein purification approaches had been unsuccessful due to instability of the purified receptors. Therefore, a combination of partial receptor purification, antibody generation and expression library screening was used to identify likely cDNA candidates for the membrane progesterin receptor [128]. Solubilization procedures that extracted most of the receptor from membranes but did not interfere with the radioreceptor assay were developed to track the receptor protein through the subsequent purification steps. The solubilized receptor preparation was fractionated on a DEAE anion exchange column and eluted with a step-wise NaCl gradient (Fig.1). Most of the [³H]20 β S binding activity eluted in the first fraction (fraction I) which contained only a single major protein band on SDS-PAGE with a molecular weight of ~40kDa and only minor amounts of other proteins, whereas fractions II and III contained many membrane proteins (Fig.1). Practically all of the receptor binding activity in fraction I was lost upon further fractionation by column chromatography. Therefore, DEAE fraction I was used to immunize mice, spleenocyte hybridomas were established, and the monoclonal antibodies secreted into the culture medium by the hybridomas were subsequently screened for their ability to detect the membrane progesterin receptor in a novel double-antibody receptor-capture assay [128]. One positive antibody, PR10-1, which detected a single 40kDa band in DEAE fraction I, was used to screen a spotted seatrout cDNA expression library prepared from ovaries containing fully grown follicles primed with gonadotropin to enhance progesterin membrane receptor expression. A positive clone, a 1.4 Kb fragment novel, was detected and completely sequenced and appeared to be a novel cDNA, unrelated to any previously described gene, which encoded a protein of ~40kDa with 352 amino acids [150]. Subsequent comprehensive studies revealed that the characteristics of this novel gene and its

protein are consistent with its identity as the membrane progesterin receptor mediating meiotic maturation of spotted seatrout oocytes.

Seven criteria were satisfied for designation of the novel seatrout gene and its protein as a membrane progesterin receptor [150]. *Plausible structure*: hydrophilicity and trans-membrane analysis of the deduced amino acid sequence predicts the protein has seven transmembrane domains, which is characteristic of the largest class of membrane receptors, G protein coupled receptors. *Tissue specificity*: Northern blot analysis showed that the mRNA of the novel seatrout receptor (mPR α) mRNA (4.0kb) was abundant in the ovaries, testes, pituitary and brain, consistent with the known regulatory roles of progestins in reproductive and endocrine functions. *Subcellular localization*: Immunocytochemistry of ovarian follicles and Western blot analysis of seatrout tissues using a polyclonal antibody directed against a peptide fragment in the N-terminal region of seatrout mPR α showed that the 40kDa protein is localized peripherally in the region of the oocyte plasma membrane, with practically no staining intracellularly and lower expression in the follicle cells surrounding the oocytes. Strong immunoreactive bands (~40kDa and 80kDa) have also been detected using the seatrout mPR α antibody in Western blots of seatrout sperm plasma membranes, where a progesterin membrane receptor has previously been characterized using radioreceptor assay techniques [120]. This localization of the seatrout mPR α on the plasma membranes of oocytes and sperm is consistent with previous biochemical binding and functional studies demonstrating the presence of membrane progesterin receptors on these germ cells mediating progesterin induction of oocyte maturation and sperm hypermotility, respectively. *Steroid binding*: Evidence was obtained that the recombinant seatrout mPR α protein can bind progestins with the characteristics of a hormone receptor. High affinity, low capacity, displaceable and specific binding for progestins typical of steroid membrane receptors was initially demonstrated with the recombinant protein produced in a bacterial (*E.coli*) expression system [150], and subsequently confirmed in membranes prepared from human breast cancer cells (MDA-MB-231 cells, nuclear progesterone receptor negative) transfected with the seatrout cDNA [122]. Recombinant seatrout and human mPR α s produced in this eukaryotic expression system display high affinity (K_d 4-8 nM), limited capacity (B_{max} 0.03-0.3 nM), displaceable progesterin binding, with rapid rates of association and dissociation (T_{1/2}: 2-5 min.) of [³H]progesterin binding to the receptor proteins [122]. Competition studies with a variety of natural and synthetic steroids demonstrate that binding to seatrout, human and zebrafish mPR α s is specific for progestins, androgens display moderate affinity for the receptors, whereas cortisol and estradiol-17 β do not bind to the receptors, even at high concentrations (Fig.2A-C). The steroid specificity of the mPR α s differs from that of the nuclear progesterone receptor because the nuclear progesterone agonist and antagonist, R5020 and RU486, show negligible binding to the seatrout and human mPR α s (Fig.2A, 2B). Importantly, the human, seatrout and zebrafish mPR α s show the highest binding affinities for the physiologically important progesterin hormone in each species, progesterone in humans, 20 β -S in seatrout and 17,20 β DHP in zebrafish. The binding of the other vertebrate progesterin hormones was at least ten-fold lower than that of the endogenous progesterin hormone for each of these receptors in transfected MDA-MB-231 cells (Fig.2). The finding that these three recombinant mPR α s produced in the same expression system have different progesterin specificities suggests that specific progesterin binding is an intrinsic property of these proteins. The demonstration that recombinant mPR proteins produced in both eukaryotic and prokaryotic systems display similar high affinity, specific progesterin binding is also consistent with this hypothesis. Different research groups have shown that recombinant zebrafish mPR β , *Xenopus* mPR β , goldfish mPR α and ovine mPR α also have the binding characteristics of progesterin membrane receptors [1,4,37,132]. *Signal transduction*: Physiological concentrations of progestins, but not estrogens and androgens, cause rapid activation of signal transduction pathways in the transfected MDA-MB-231 cells. Treatment with 20 β -S causes a decrease cAMP levels which is reversed by pretreatment with activated pertussis toxin (PTX), but not after pretreatment with inactive PTX, suggesting seatrout

mPR α activates a PTX-sensitive inhibitory G protein [150] which has been confirmed in a subsequent study [122], consistent with its identity as the MIS receptor. Similarly, evidence has been obtained that progesterone treatment down-regulates adenylyl cyclase activity through an inhibitory G protein via both recombinant and wild type human mPR α and mPR β in several cell models [21,41,122]. *Hormonal regulation:* The upregulation of the seatrout mPR α protein observed in response to hormonal treatment further suggests an involvement of the receptor in oocyte maturation. Induction of oocyte maturation in seatrout ovarian fragments incubated with gonadotropin was accompanied by an increase in seatrout mPR α protein expression, in agreement with the earlier radioreceptor studies showing membrane progesterin receptor concentrations are increased by this treatment and are associated with the development of oocyte maturational competence [125]. *Biological relevance:* The pattern of seatrout mPR α expression during the reproductive cycle also suggests it is the MIS receptor. The concentrations of seatrout mPR α are elevated in fully grown oocytes and show a further increase during oocyte maturation in fish captured on their spawning grounds [150], similar to the pattern of changes in 20 β S receptor binding activity during oocyte maturation described previously [125]. Spotted seatrout oocytes are relatively small and fragile and do not survive for extended period (16-18hrs) after microinjection. Consequently, experiments to examine the requirement for seatrout mPR α in oocyte maturation by microinjection of antisense oligonucleotides could not be conducted. Instead, antisense oligonucleotides to the homologous mPR α identified in zebrafish, were injected into zebrafish oocytes which are larger and can survive for an extended period after microinjection. Injection of both phosphothioate and morpholino antisense oligonucleotides, but not sense or mis-sense ones, during gonadotropin induction of priming blocked the subsequent response of zebrafish oocytes to the zebrafish MIS, 17,20 β DHP, most of them failing to mature [150]. The involvement of mPR α in oocyte maturation has also been demonstrated goldfish oocytes using the same experimental approach [133]. It is concluded from all these studies that mPR α has all the characteristics of a membrane progesterin receptor and is the MIS receptor in spotted seatrout.

3. Identification and characteristics of mPRs in other species

Soon after the discovery of the seatrout mPR α cDNA, thirteen closely-related genes were identified in other fish, amphibian and mammalian species [151]. The mPRs have been classified into three subtypes, α , β , and γ , all of which are present in both fish and mammals, including humans [151]. The subcellular localization, progesterin binding, G protein coupling and signaling characteristics of recombinant human mPR α produced in an eukaryotic expression system are very similar to those of seatrout mPR α [122]. Recombinant zebrafish, goldfish and ovine mPR α s expressed in mammalian cell lines have also been shown to have the binding characteristics of membrane progesterin receptors [1,37,132]. Equivalent information on the steroid binding and signaling characteristics of the β and γ subtypes in most of these species is lacking, although the limited data currently available for human, zebrafish and *Xenopus* mPR β s suggest they are similar to those of the mPR α s [4,37,41]. However, the finding that the α , β and γ subtypes have different tissue distributions and expression patterns throughout the reproductive cycle in a variety of animal models [15,29,42,79] suggests they may have different physiological functions to those described in the following section for mPR α . The mPRs belong to the seven-transmembrane progesterone adiponectin Q receptor (PAQR) family which has a different origin than that of the GPCR superfamily [115,122]. Phylogenetic and sequence analyses show that another type of PAQR, PAQR 6, is closely related to the mPRs and may be an additional member of this progesterin receptor subfamily [122]. Another PAQR type, PAQR 9, may also be a member of the mPR subfamily because it is clustered with the mPRs [122].

4. Tissue distribution of mPR α

The broad distribution of mPR α mRNAs in reproductive and non-reproductive tissues suggests they have diverse physiological functions in vertebrates. In the initial publication mPR α mRNA was only identified in spotted seatrout ovaries, testes, brain and pituitary by Northern blot analysis [150]. Similarly, mPR α expression was found to be restricted to the gonads in zebrafish [42]. However, it was shown in channel catfish with a more sensitive real-time PCR procedure that in addition to high expression in these tissues, mPR α transcripts were also present on all the nonreproductive tissues examined, with relatively high expression throughout the brain and in the muscle and heart, and low levels of expression in gill, intestine, liver, kidney, interrenal, muscle and spleen [42]. Similarly human mPR α mRNA was initially identified primarily in reproductive tissues, particularly in the ovary, testis, placenta and uterus, and also in the kidney and adrenal by dot blot hybridization [150]. Recent studies have also identified mPR α in human myometrium [15,29,41], amnion, chorion and endometrium [29], breast tissues [23], and lymphocytes [21] by RT-PCR. Expression of mPR α mRNA has also been demonstrated in the sheep hypothalamus, pituitary, ovary and corpus luteum [1], the corpus luteum of rats [13] and in the mouse brain, testis and uterus [119]. Moreover, changes have been observed in the expression of mPR α mRNA in many of these tissues during the reproductive cycle and in malignant human breast tissues, which is consistent with important physiological roles for the receptor in a wide variety of reproductive functions as well as its potential involvement in tumorigenesis. However, to date only a few of these possible functions of mPR α have been investigated and are discussed below.

Functions of mPR α

1. Progesterin induction of oocyte maturation in fish and amphibian oocytes

Fully grown oocytes of fish and other vertebrates cannot be fertilized because the first meiotic division is incomplete and is arrested at the G2/M border of prophase I. Meiosis is arrested by high intra-oocyte levels of cyclic AMP which are maintained by adenylyl cyclases [19,36]. A surge in gonadotropin secretion induces the resumption of meiosis in fish and amphibian oocytes through an intermediary, a MIS, which is secreted from the ovarian follicle cells in response to gonadotropin stimulation [59,75,146]. The MIS in turn binds to a specific receptor on the oocyte plasma membrane to initiate a process called oocyte maturation via a nongenomic mechanism, resulting in a fully mature oocyte capable of being ovulated and fertilized [75, 117]. As mentioned previously, the hormonal control of oocyte maturation in these two vertebrate groups has been one of the most thoroughly investigated models of nongenomic steroid actions, since it was first described more than twenty years ago. The MISs in fishes have been positively identified as novel progesterin hormones with multiple hydroxyls on the sidechain, 17,20 β DHP in salmonids and cyprinid fishes such as goldfish and zebrafish [74], and 20 β -S in advanced marine perciform fishes such as Atlantic croaker and spotted seatrout [126,134]. In contrast, considerable controversy surrounds the identity of the MIS in amphibians such as *Xenopus*, because in addition to progesterone, androgens are also potent inducers of oocyte maturation in this species [54]. Several lines of evidence indicate that MIS induction of oocyte maturation in fish is via a nonclassical steroid mechanism because it is rapid, nongenomic and initiated at the cell surface by binding to progesterin membrane receptors [20,73,121] which have been characterized in several fish species (see earlier section). The MIS initiates oocyte maturation in fish by causing a decline in intracellular cAMP levels [35, 39], which is associated with activation of a PTX-sensitive inhibitory G protein pathway [128,148]. Direct evidence was obtained in spotted seatrout that MIS binding to the progesterin membrane receptor causes activation of a PTX-sensitive inhibitory G protein pathway which is necessary for the completion of oocyte maturation [83]. Cyclic AMP production in seatrout oocyte membranes, an indirect measure of adenylyl cyclase activity, was specifically down-regulated by 20 β -S, whereas cortisol and testosterone were ineffective, and this action of

20 β -S was blocked by PTX. The finding that 20 β -S induction of oocyte maturation was blocked by microinjection of PTX into the oocytes is also consistent with 20 β -S activation of a G $_i$ protein. Confirmation that 20 β -S activates a G protein was shown by increased binding of [35 S]GTP γ S to ovarian membranes after treatment with the steroid, and a follow up immunoprecipitation experiment demonstrated that the increased [35 S]GTP γ S binding was associated with the alpha subunits of inhibitory G (G $_{i1-3}$) proteins. Uncoupling of G proteins from GPCRs with excess guanine nucleotides such as GTP γ S and inhibitory G proteins with PTX decreases ligand affinity of the receptors [16,137]. Binding of [3 H]20 β -S to seatrout ovarian membranes was decreased after treatment with either GTP γ S or PTX [83], suggesting that the seatrout membrane progesterin receptor is directly coupled to an inhibitory G-protein. On the basis of these results it was proposed that 20 β -S activation of an inhibitory G protein results in down regulation of adenylyl cyclase activity in spotted seatrout oocytes through the α subunit of the G protein leading to a reduction in protein kinase A (PKA) activation [83]. In Atlantic croaker, a marine fish belonging to the same family as spotted seatrout, 20 β -S induction of oocyte maturation is also associated with a decrease in cAMP levels [82]. However, the cell permeable PKA inhibitors were ineffective in inducing or enhancing 20 β -S-induced oocyte maturation in this species [82]. On the other hand inhibitors of phosphodiesterase activity partially blocked 20 β -S induction of oocyte maturation in this species and inhibitors of the phosphatidylinositol 3-kinase (PI3K)/Akt signal transduction pathway completely blocked the response to 20 β -S [82]. Pace and Thomas proposed that the $\beta\gamma$ subunits of the heterotrimeric G protein recruit PI3K γ to the plasma membrane to catalyze the formation of phosphatidylinositol-3,4,5-trisphosphate (PIP3), which in turn binds to serine/threonine kinase Akt resulting in its activation and subsequent alteration of phosphodiesterases (PDEs), which breakdown cAMP [82]. Therefore, binding of the MIS, 20 β -S to its receptor and activation of an inhibitory G protein could result in a decline in intraoocyte cAMP levels through multiple signaling pathways to release the oocytes from meiotic arrest and complete maturation.

Considerable evidence has been obtained in the past few years which strongly suggest that mPR α is the primary progesterin membrane receptor mediating MIS induction of oocyte maturation in fishes. The initial study on mPR α expression in seatrout showing the protein is localized mainly on the oocyte plasma membrane and increases in abundance during the reproductive cycle and during natural and gonadotropin-induced oocyte maturation is consistent with its involvement in this process [150]. The signal transduction pathway activated by recombinant seatrout mPR α is identical to that described in seatrout oocytes, because it has been shown that the recombinant seatrout mPR α protein activates an inhibitory G protein and is directly coupled to it, resulting in down-regulation of cAMP production (adenylyl cyclase activity) via a PTX-sensitive pathway [150,122]. Moreover, a co-immunoprecipitation experiment confirms that the mPR α protein on seatrout plasma membranes is coupled to an inhibitory G protein (Fig. 3). More direct evidence for a role for mPR α in oocyte maturation in the original study was obtained in zebrafish, in which microinjection of antisense oligonucleotides into oocytes during the initial gonadotropin priming period blocked the completion of oocyte maturation in response to the MIS, 17,20 β DHP [150]. An important role for mPR α in oocyte maturation was subsequently confirmed in another fish model, goldfish, using the same experimental approach [133]. Microinjection experiments into zebrafish oocytes with antisense oligonucleotides to mPR β suggest it is also involved in oocyte maturation in this species [124]. Finally, a close correlation between the binding affinity of a wide range of steroids to recombinant mPRs and their ability to induce oocyte maturation has been observed. For example, an unusual ligand for goldfish mPR α , diethylstilbestrol, was shown to be effective in inducing oocyte maturation in this species, and the relative binding affinities of diethylstilbestrol analogs for the receptor correlated well with their oocyte maturation inducing activities in an *in vitro* bioassay [132]. Taken together, these studies on the role of mPR α in oocyte maturation in fish provide the most compelling evidence obtained to date for an important function for mPRs in the hormonal control of a physiological process.

Interestingly, attempts to clone the mPR α from *Xenopus laevis* cDNA libraries have been unsuccessful which suggests this mPR subtype may be absent in this species [4,150]. A recent study suggests the β homolog, which is expressed on the plasma membrane of *Xenopus* oocytes and shows specific progesterone binding when expressed in mammalian cells, has a similar role in the progestin induction of oocyte maturation in this amphibian model as that demonstrated for mPR α in teleosts [4]. Microinjection of a specific mPR β antibody into *Xenopus* oocytes inhibited progesterone-induced oocytes maturation, whereas oocyte maturation was accelerated after microinjection *Xenopus* mPR β mRNA [4].

2. Progestin stimulation of sperm hypermotility in fish and humans

Nongenomic steroid actions can be examined alone in vertebrate sperm because they are considered to be transcriptionally inactive. Rapid actions of progesterone to induce both the acrosome reaction and stimulate hypermotility in mammalian sperm have been demonstrated in several laboratories and a variety of receptors have been proposed as potential mediators of these multiple progesterone effects [6,11,103,108,138]. The mechanism of progestin stimulation of sperm hypermotility can be studied in isolation in fish sperm without the complication of progestins activating pathways involved with the acrosome reaction which does not occur in fish. Rapid induction of sperm hypermotility by progestin hormones and the presence of specific progestin receptors on their sperm membranes have also been demonstrated in several fish species. The fish progestin, 20 β -S, causes a concentration-dependent (10 to 200nM) increase in the motility of croaker sperm after a 5-minute incubation [124] which is associated with rapid increases in intrasperm free calcium and cAMP concentrations [118]. High-affinity ($K_d \sim 20$ nM), low capacity ($B_{max} \sim 0.08$ nM) single binding sites specific for 20 β -S have been demonstrated on croaker and spotted seatrout sperm membranes with binding characteristics similar to that of recombinant seatrout mPR α [120,122,127]. Moreover, mPR α proteins have been identified on the sperm membranes of these two species and localized to their midpieces, consistent with an involvement of the receptor in sperm motility [127, 136,150]. Recent studies on both species demonstrating that sperm with low motility show decreased expression of mPR α protein in their sperm membranes further support a physiological role for mPR α in progestin-induced sperm hypermotility [135]. In agreement with the results of experiments in other animal models and cell types expressing mPR α , progestin treatment causes G protein activation in sperm membranes and the receptor co-immunoprecipitates with a G protein, suggesting mPR α functions as a GPCR in sperm [135]. However, in contrast to these other studies, progestins increase cAMP production in croaker sperm membranes, suggesting activation of a stimulatory G protein. This signaling pathway is critical for progestin induction of sperm hypermotility because it is completely blocked by pretreatment with two specific inhibitors of membrane adenylyl cyclases [135]. Immunoprecipitation studies with specific G protein α subunit antibodies showed an olfactory G protein (G_{olf}) was specifically activated by progestins, and immunocytochemical analysis localized G_{olf} on the midpiece, the same region of sperm where mPR α is expressed. Finally, it was demonstrated that mPR α is co-immunoprecipitated with the G_{olf} antibody, suggesting the receptor is coupled to this G protein. It is concluded from these studies that mPRs can couple to a variety of G proteins in different tissues.

Currently, only limited information is available on the physiological and signaling functions of mPR α in mammalian sperm. The mRNA for mPR is present in the human and mouse testis [151,119] and the protein has been detected on human sperm membranes [unpubl. obs.]. Progesterone effects on the acrosome reaction in mammalian sperm can occur independently from its effects on hyperactive motility and involve different intracellular mechanisms [38, 58], suggesting they may be mediated via different receptors. Immunofluorescence analysis of human sperm with a mPR antibody shows that the receptor the mPR is localized primarily to the midpiece and is not detected on the acrosome, consistent with a role for the receptor in

sperm motility. Furthermore, sperm from human donors with low motility had significantly lower mPR α protein concentrations than that in normal motile sperm. Finally, it was demonstrated that G proteins were activated in human sperm in response to progesterone treatment [unpubl. obs.]. Taken together, these preliminary results suggest that mPR α may have a similar function in promoting hypermotility in mammalian sperm to that proposed for fish sperm.

3. Progesterone signaling in human myometrial cells

Although one of the characteristics of parturition in most mammals is the dramatic drop of progesterone levels prior to labor, maternal progestin levels do not decrease during parturition in humans and some primates. Instead, progesterone production by the human placenta gradually increases with advancing pregnancy, reaching 300mg per day at term [140,147]. Given the relaxant effects of progesterone, for human labor to proceed there must be some form of progestin withdrawal. The expression of nPR-responsive genes is decreased in the primate uterus at term [17], which suggests “functional” progestin withdrawal involves repression of nPR transcriptional activity, although the mechanism remains unclear. One possible intermediary in this withdrawal is the truncated nPR isoform, PR-A, which is upregulated in laboring myometria and can function as a “dominant negative” to repress the transcriptional activity of the PR-B [94,139]. Karteris and coworkers examined another possibility, that progestins also activate mPRs at the cell surface to alter PR-B transcriptional activity via a nonclassical mechanism of steroid action. Their investigation on the signaling and functions of mPR α and mPR β in human myometrial cells in culture is the most comprehensive investigation to date on the physiological roles of mPRs in a mammalian model [41].

Transcripts for mPR α and mPR β were identified by real-time PCR in lower myometrial tissue from pregnant women in labor and in subcultured myometrial cells and both mPR proteins were detected on myometrial cell plasma membranes by western blotting and immunocytochemistry [41]. The presence of specific progestin binding on the cell surface of the myocytes was confirmed with a fluorescent probe, progesterone-BSA-FITC, in the presence of steroid competitors and also by radioreceptor assay. This specific progesterone binding was attributed to the presence of mPR α and mPR β , because it was significantly reduced in cell membranes prepared from myocytes that had been transfected with siRNAs to both receptors. Steroid competition assays and signal transduction studies confirmed that mPR α and mPR β are functional progestin receptors on the cell surface of human myocytes. The progesterone binding was not due to the presence of nuclear progesterone and glucocorticoid receptors, since R5020 and dexamethasone did not displace [3 H]-progesterone binding from the membrane preparations. Progesterone treatment of myocyte membranes caused activation of inhibitory G proteins and the resulting decline cAMP production through a pertussis toxin-sensitive pathway. Interestingly, a variety of inhibitory G proteins, Gi $_{1/2}$, Gi $_3$ and Go, were activated in response to progesterone treatment, whereas there was no activation of Gs and Gq $_1$. In agreement with the steroid binding studies, G protein activation by steroids was specific for progesterone and R5020, dexamethasone and cortisol were ineffective. Finally, both mPR α and mPR β were shown to directly interact with inhibitory G proteins in two co-immunoprecipitation assays, suggesting they function as GPCRs. The results are identical to those obtained with recombinant mPR α in a eukaryotic expression system [122].

Possible cross-talk between the mPRs and nPR was examined by assessing the binding of the nPR to a glucocorticoid-responsive element (GRE) linked to a luciferase reporter vector, which was transiently transfected into myocytes [41]. As predicted, progesterone treatment with progesterone, dexamethasone and the nPR agonist, R5020, caused transactivation of PR-B in myocytes with high levels of PR-B expression. However, pertussis toxin treatment, which

prevents inhibitory G protein signaling through mPRs, differentially affected the PR-B transactivation response to the three steroids. Pretreatment with the toxin partially blocked the response to progesterone, whereas the responses to dexamethasone and R5020, which do not activate mPRs, were unaltered. Additional stronger evidence of an involvement of mPRs in progesterone transactivation of PR-B was obtained from experiments in which myocytes were also transiently transfected with siRNAs for mPR α and mPR β . The transactivation responses to progesterone were greatly attenuated in myocytes treated with siRNAs for each of the mPRs compared to that in myocytes with normal mPR levels. It was concluded from these studies that progesterone acts through a mPR-dependent pathway as well as through PR-B to transactivate the nPR. Moreover, the results showed that the potentiation of PR-B transactivation through mPRs involves activation of a pertussis-sensitive inhibitory G protein. On the basis of these results, Karteris et al. proposed that during early pregnancy progesterone activation of mPRs results in an amplification of the PR-B action to maintain the myometrium in a quiescent state [41]. Importantly, these results also provided the first evidence that novel steroid membrane receptors can influence the transactivation activities of nuclear receptors.

The mPRs can also potentially influence the transactivation of PR-B in human myometrial cells collected at term through altering the expression of co-activators. The decrease observed in co-activator expression in human myometria during labor has been proposed to contribute to the initiation of partition by impairing nPR function [17]. Expression of the mPRs appears to be elevated at this stage of pregnancy which is likely associated with increases in circulating progesterone and estradiol-17 β levels, since mPR mRNA and protein levels are upregulated in myocytes treated with these steroids *in vitro*. Interestingly, the two mPRs are differentially regulated by these steroids; both are upregulated by estradiol-17 β , whereas only mPR α is upregulated by progesterone. Treatment of these myocytes with a progesterone-BSA conjugate *in vitro* caused transient downregulation of SRC2 mRNA and protein expression which had returned to pretreatment values 1-2 days later, whereas the levels of other co-activators were not significantly altered (Fig.4). This response to progesterone-BSA was attenuated in cells that had been transfected with siRNA for mPR α but not mPR β , which suggests the progesterone-induced downregulation of the SRC2 gene is at least partially mediated through mPR α via an unknown mechanism. This downregulation of SRC2 expression through mPR α could potentially contribute to the functional progesterone withdrawal in human myometria during labor [41].

Other progesterone signaling pathways initiated at the cell surface of human myocytes through the mPRs were identified that could also facilitate myometrial contraction. Phosphorylation of myosin light chain (MLC) at Ser¹⁹ is the primary mechanism regulating the contractile status of smooth muscle cells [25]. Cyclic AMP inhibits phosphorylation in these cells causing muscle relaxation. Activation of inhibitory G proteins through the mPRs, as predicted, caused rapid decreases in basal and isoproterenol-induced cAMP production *in vitro* by cultured myocytes and membranes prepared from pregnant myometrial biopsies, which was blocked by pretreatment with pertussis toxin. Therefore, progesterone via the mPRs could potentially remove an inhibitory influence of cAMP on MLC phosphorylation by decreasing cAMP levels. Clear evidence of an involvement of mPRs in MLC phosphorylation was obtained from experiments showing that treatment of myocytes with progesterone-BSA induced phosphorylation of MLC at Ser¹⁹ and that this progesterone action was blocked by pretreatment with pertussis toxin and by transfecting the cells with siRNA for mPR α [41]. Evidence for the presence of a stimulatory progesterone pathway to phosphorylate MLC involving phosphorylation of p38 MAPK was obtained in a subsequent study. The demonstration that pretreatment with pertussis toxin blocked the phosphorylation of p38 MAPK in response to progesterone-BSA suggests this action is mediated through mPR/Gi. Moreover, the finding that treatment with a specific inhibitor of p38 MAPK, SC-68376, almost completely blocked phosphorylation of MLC, suggests that phosphorylation of p38 MAPK is

the primary mechanism regulating MLC phosphorylation. Taken together, these studies suggest that progesterone signaling through mPRs could promote myometrial contraction at the onset of labor through three mechanisms; by down-regulating SRC2, which together with a decreased PR-B/PR-A ratio, results in a decrease in PR-B transactivational activity; by inhibiting adenylyl cyclase; and by inducing phosphorylation of MLC [41].

4. Progesterone signaling in human T lymphocytes and Jurkat cells

There is a growing body of evidence that suggests progesterone modulates immune function in mammals [21]. For example, changes in the symptoms of autoimmune diseases such as rheumatoid arthritis, in the cellular immune response to infection, and in the expression of interferon-related genes have been observed in women during the menstrual cycle and pregnancy in [22,40,76]. Progesterone has also been shown to promote T cell differentiation *in vitro* resulting in alterations in the production of cytokines associated with humoral immunity [93]. However, the majority of studies have failed to detect the nPR in immune cells [2,57, 114], which have led researchers to propose alternative mechanisms of progesterone action [24].

Transcripts for mPR α and mPR β were recently identified in peripheral blood leukocytes and T lymphocytes of reproductive age women and in immortalized human T (Jurkat) cells, whereas nPR and mPR γ were not detected in any of these cell types [21]. Western blot analyses confirmed expression of mPR α and mPR β proteins on the plasma membranes of human T cells and Jurkat cells. Interestingly, mPR α mRNA expression was upregulated more than two-fold during the luteal phase of the menstrual cycle in the CD8⁺ T lymphocytes, but not in the CD4⁺ T lymphocytes. Dosiou et al., proposed that this increase in mPR α expression could potentially contribute to the immunomodulatory effects of progesterone during the second half of the menstrual cycle and in pregnancy. In support of this they cited a study in mice which showed that CD8⁺ T lymphocytes mediate the protective effects of progesterone against stress-induced abortion by altering the cytokine profile to the production of those associated with humoral immunity [7].

Clear evidence was obtained for the presence of functional membrane progesterone receptors and progesterone activation of G proteins on human T cell and Jurkat cells [21]. Saturation and Scatchard analyses of [³H]-progesterone binding to Jurkat membrane showed the presence of a high affinity, single binding site with a K_d of 4.6 nM, very similar to the K_d (4.17 nM) reported for recombinant mPR α in human breast cancer cells [122]. Progesterone caused G protein activation in Jurkat cell membranes in a concentration-dependent manner, whereas the nPR agonist R5020 and a GR agonist, dexamethasone, were ineffective. An immunoprecipitation experiment with antibodies directed against the alpha subunit of different G proteins revealed that progesterone activated an inhibitory G protein in the immortalized T cells, consistent with previous studies showing that mPR α is coupled to and activates an inhibitory G protein. Taken together these results strongly suggest that progesterone acts on T cells through mPRs resulting in activation of an inhibitory G protein pathway. The functional consequences of activation of this pathway, however, remain unclear.

5. Progestin down-regulation of GnRH secretion in fish and rodents

Only circumstantial evidence has been obtained to date for a role of mPR α in progestin negative feedback of gonadotropin releasing hormone (GnRH) secretion in vertebrates. Both the mPR α mRNA and protein are expressed in the brains of spotted seatrout and Atlantic croaker [150,81]. Treatment of preoptic anterior hypothalamic (POAH) tissue slices excised from adult Atlantic croaker at the beginning and at a midpoint of the reproductive cycle *in vitro* with the fish progestin, 20 β -S (290 nM) caused a rapid decrease in the secretion of seabream GnRH, the hypophysiotropic GnRH in this species [124]. The release of GnRH into the incubation

medium declined to less than 20% that of controls within the initial 10-minute incubation period and was also significantly decreased during longer incubation periods. The physiological significance of this rapid negative feedback effect of 20β -S on GnRH secretion is unclear because experiments have not been conducted with POAH tissues collected from croaker at the end of the reproductive cycle during oocyte maturation and spermiation when plasma 20β -S levels are elevated. However, the previous finding that 20β -S exerts a similar negative feedback effects on GnRH content in the POAH and LH secretion at the midpoint of the reproductive cycle as at the end of the cycle [60], suggests that these rapid effects are also manifest at the end of the cycle. The feedback mechanism may switch off LH secretion once production of 20β -S and maturation of the largest most responsive cohort of oocytes are underway, to prevent over stimulation and precocious maturation of the remaining smaller, less developed oocytes. Additional studies using specific mPR α agonists will be required, however, to clearly establish a role for mPR α in mediating these rapid feedback effects of progestins on GnRH secretion in teleosts.

Similarly, only indirect evidence has been obtained so far implicating mPR α in progesterone feedback effects on GnRH secretion in mammals. The existence of rapid progesterone actions on reproductive neuroendocrine functions in rodents has been known for two decades [43] and specific progesterone binding sites have been detected on brain membrane preparations [131]. Both mPR α and mPR β subtypes are present in the preoptic anterior hypothalamic region of the rodent brain and in immortalized GnRH-secreting neurons, GT1-7 cells. The finding that rapid down regulation of luteinizing hormone secretion by progesterone also occurs in PRKO mice indicates it is mediated by a novel progesterone receptor [Sleiter, Levine, pers. com.].

Progesterone membrane receptor component 1-PGMRC1

1. Progesterone binding sites on mammalian liver membranes

Progestin binding moieties have been detected in microsomal membranes prepared from rat and porcine livers [64,145]. Partial purification of the microsomal membranes by anion-exchange chromatography produced two protein fractions with apparent molecular weights of 28 and 56 kDa with progesterone binding activity. Subsequent amino acid sequencing of the amino terminus of the two protein fractions revealed that they are identical, suggesting that they represented monomers and dimers of the same protein [64]. Receptor binding assays were conducted with both microsomal and solubilized membrane preparations. Both high affinity (K_d 11 nM) and low affinity (286 nM) binding sites for progesterone were identified in the microsomal protein fractions. The kinetics of association and dissociation of [3 H]-progesterone binding to the both the microsomal and solubilized membrane fractions are rapid, with $t_{1/2}$ s of 3-8 minutes [64]. In single point competition assays progesterone displays the highest affinity of the compounds tested, with an IC_{50} of 0.5 μ M followed by corticosterone which has an approximate relative binding affinity (RBA) 25 % that of progesterone and an IC_{50} of 2 μ M. Testosterone and cortisol also are effective competitors for [3 H]-progesterone binding, with IC_{50} s of (RBAs 16% and 4%) respectively, whereas estradiol-17 β has no affinity for the progesterone binding site. Such high relative binding affinities of corticosteroids for a progesterone binding protein are unique and may indicate that the putative receptor is activated by corticosteroids rather than progesterone, because plasma corticosteroid levels are higher than progesterone levels under many physiological conditions [64]. It was suggested, on the basis of a subsequent study showing that sigma (σ) ligands such as haloperidol, carbetapentane citrate and Rimeazole compete with [3 H]-progesterone binding to the porcine liver membrane preparations, that the progesterone binding sites are related to the σ receptor binding site superfamily [65]. However, no follow up studies have been conducted to test the binding of σ ligands to PGMRC1 so the significance of these findings remains unclear.

2. Cloning, identification and characterization of porcine PGMRC1

A cDNA was cloned from porcine smooth muscle cells with a deduced amino acid terminal sequence identical to that of the progesterone/corticosterone binding moiety previously purified from porcine liver tissue [27,64]. The cDNA encodes a protein with 194 amino acids and is now known as progesterone membrane receptor component one (PGMRC1) [12]. The binding characteristics of recombinant PGMRC1 fused to glutathione S transferase (GST) produced in a prokaryotic expression system (*E.coli*) were investigated. Recombinant PGMRC1 (named IZA1) was incubated with [3H]-P4 for two hours, GSH-sepharose was added, and a GST pull-down assay performed. No specific [3H]-progesterone binding was detected, whereas the recombinant protein was able to bind heme, suggesting a functional protein was produced by this recombinant expression system [67]. Moreover, addition of IZA1 antibody to a mixture of [3H]-P4 and recombinant IZA1 did not show any specific isotope binding [67]. In contrast, stable transfection of PGMRC1 into CHO cells [26] and over expression in spontaneously immortalized granulosa cells (SIGCs) caused an increase in specific [3H]-progesterone binding to the microsomal fraction [91]. Single point competition assays showed that whereas progesterone showed highest affinity for the receptor (IC50s 0.5 μM), testosterone and corticosterone also competed for progesterone binding, with IC50s of approximately 1 μM and 10 μM, respectively. There are several plausible explanations for the differences in the ability of recombinant PGMRC1 produced in the prokaryotic and eukaryotic expression systems to bind progesterone, including deficiencies in protein expression or the lack of another protein(s) in the *E. coli* system which are present in eukaryotic cells and forms a complex with PGMRC1 that is required for progesterone binding.

In contrast to the extensive studies on PGMRC1, there are very few reports on the closely human gene PGMRC2 which was cloned by Wehling and coworkers [33]. PGMRC2, together with PGMRC1, is expressed on human sperm and has been suggested to be involved in progesterone-induced acrosome reaction [51].

2. Characteristics of PGMRC1

Progesterone membrane receptor component 1 is a vertebrate 26-28 kDa protein with a single transmembrane domain and a potential internal cytochrome *b5* binding sequence. PGMRC1 and the closely-related PGMRC2 have homologues belonging to the gene family named membrane associated progesterin receptor (MAPR) in other eukaryotes, including nematodes, insects, yeast and *Aripidopsis* [12]. The gene has been cloned independently in vertebrates by several research groups and has been given several other names related to the diverse biological processes or tissues it has been associated with, such as its induction by dioxin (25-Dx0 [110], homology of a human gene to the porcine progesterone receptor (Hpr6) [33], a rat 195 amino acid protein (ratp28) [77], and 75 amino acid truncated form of ratp28 (HC5) [48], embryogenesis (VemaA) [107], and inner zone antigen in the adrenal cortex (IZA) [100]. A bewildering variety of ligands have been shown or suggested to bind to PGMRC1, including heme [68,112], cholesterol [62] and steroids with 21 carbons, glucocorticoids and progestins [12]. A corresponding wide range of physiological roles have been suggested for this protein such as steroid synthesis and metabolism [100], cholesterol regulation, axonal guidance [107], endocytosis, alteration of reproductive behaviors [46], a component of a progesterone receptor and as an adapter protein. Recently evidence has been obtained in several laboratories that PGMRC1 forms complexes with other proteins. Complexes have been identified with proteins believed to be involved in the cholesterol system, SCAP and Insig1, in cells co-transfected with PGMRC1 and either of these proteins [113], consistent with a possible role for PGMRC1 in cholesterol regulation [12]. Only the proposed progesterone receptor functions of PGMRC1 will be discussed here.

Functions of PGMRC1

Antiapoptotic progesterone actions on ovarian follicle cells

Progesterone has an important physiological role in the ovarian follicle to inhibit apoptosis of granulosa and thecal cells, but the mechanism of progesterone action remains unclear because the nuclear progesterone receptor is not expressed in rat thecal cells and only transiently expressed in granulosa cells [86,116]. Progesterone has been shown to act at the cell surface to inhibit apoptosis in these cells, suggesting it is acting through a membrane progesterone receptor [87,88,97,98]. PGMRC1 has been detected in rodent luteal and granulosa cells [13, 61] and its potential role in mediating these anti-apoptotic actions of progesterone in (SIGCs) have been investigated in detail by Peluso and collaborators [91]. These investigators showed that over expression of PGMRC1 in SIGCs is associated with increases in [³H]-progesterone binding with cell survival and that the anti-apoptotic action of progesterone was abrogated by pretreatment with an antibody to the N-terminal of PGMRC1 [91]. Earlier it had been demonstrated that PGMRC1 interacts (immunoprecipitates) with plasminogen activator inhibitor RNA binding protein -1 (PAIRBP-1) which is present in rat granulosa and thecal cells [90]. Previously PAIRBP-1, which had been named RDA288, had been implicated in mediating progesterone's apoptotic actions in these cells [89]. Peluso and coworkers demonstrated that both PGMRC1 and PAIRBP-1 are localized on plasma membrane of SIGCs [91]. Information on the signal transduction pathways activated by progesterone through this PGMRC1/PAIRBP-1 complex should provide clues of how the antiapoptotic actions of the steroid are mediated.

Progesterone initiation of the acrosome reaction in mammalian sperm

The large number of putative progesterone receptors that have been identified on mammalian sperm using radioreceptor, immunological and biochemical approaches has complicated investigations of their roles in progesterone actions such as induction of the acrosome reaction and hyperactivation [6,51,52,53,58,102,103]. Wehling and coworkers identified The nPR and also PGMRC1 and PGMRC2 were identified on human sperm by researchers in the Wehling laboratory [51]. These researchers also showed that the rapid increase in intrasperm calcium concentration in response to progesterone was significantly reduced after incubation of sperm with an antibody to porcine PGMRC1 (formerly named mPR) compared to that observed after incubation with pre-immune serum [26]. Previously, immunostaining had been detected on the head of human sperm using this antibody [11]. Moreover, the progesterone-initiated acrosome reaction was inhibited 60% by the antibody, suggesting that PGMRC1 is involved in the acrosome reaction [11].

c. Summary of progestin signaling pathways through mPR α and PGMRC1

Progestins have been shown to activate a variety of signaling pathways through mPR α and PGMRC1 (Fig.5). In the majority of cell models investigated to date, progestin binding to mPR α alters second messenger pathways through activation of pertussis toxin-sensitive inhibitory G-proteins. Progestins activate mitogen activated protein kinases (MAPK) causing Erk 1/2 phosphorylation in a dose-dependent fashion in nPR negative cells transfected with seatrout mPR α [150] and with zebrafish mPR α and mPR β [37], presumably through the $\beta\gamma$ subunits of the G protein (Fig. 5, pathway 1). Another MAPK, p38, is activated in human myometrial cells through mPR α , resulting in phosphorylation of myosin light chain protein in these cells [41] (Fig. 5, pathway 2). A third signaling pathway likely initiated by the G protein $\beta\gamma$ subunits through mPR α in Atlantic croaker oocytes is the Akt/PI3 kinase pathway [82] (Fig. 5, pathway 3). Progestin stimulation of the mPR α causes a decrease in cAMP levels which probably mediated by the inhibitory G protein α subunit, resulting in inhibition of adenylyl cyclase activity [21,41,83,150] (Fig. 5, pathway 4). However, a stimulatory olfactory G protein appears to be activated upon progestin binding to mPR α in croaker sperm causing an increase

in cAMP production [135] (Fig.5, pathway 5). Activation of a PTX-sensitive pathway through mPR α and mPR β in human myometrial cells alters nPR transactivation activity [41] (Fig.5, pathway 6). Activation of this pathway in human myometrial cells also altered expression of the nuclear receptor coactivator, SRC-2 [6] (Fig. 5, pathway 7). There is evidence that progestins initiate calcium mobilization from intracellular stores through both mPR α and PGMRC1 [1,26] (Fig.5, pathway 8). It is likely that additional signaling pathways mediated by these receptors will be identified when other target cells are investigated.

Methodological problems in expressing and characterizing membrane progestin receptors

a. Membrane localization of receptors

Most detailed studies of the characteristics of hormone membrane receptors have been conducted on GPCRs. The level and dynamics of GPCR expression on the cell surface are highly regulated and strongly influence the magnitude and duration of the physiological response upon ligand activation. After the receptor proteins are synthesized in the endoplasmic reticulum (ER), they undergo post-translational modifications such as folding with the aid of chaperone proteins. Defective proteins are detected by the cellular quality control system and selected for retention in the ER for recycling or destruction. Some GPCRs, such as the human GnRH receptor, rat LH receptor and the δ opioid receptor display weak expression at the plasma membrane and a large proportion of the synthesized protein is retained in the cytoplasm or in ER for degradation [18,92,95]. Conn and coworkers provide evidence that post-translational folding of the human GnRH receptor protein and its dimerization are important mechanisms controlling expression of the receptor on the plasma membrane [18]. Another major process regulating the cell-surface expression of GPCRs is their endocytic trafficking following activation [69]. As a consequence high levels of GPCR expression are rarely observed in the plasma membrane and instead the receptors show highest expression in the cytoplasm [129]. The receptors are phosphorylated by GPCRkinases enabling them to bind to arrestin which results in their desensitization. The GPCRs are subsequently targeted to endocytic sites on the plasma membrane such clathrin-coated pits where they are internalized for subsequent degradation or recycling by lysosomes.

It is not known what features of GPCR trafficking are shared by mPR α and PGMRC1. Although mPRs function as G protein coupled receptors, they have a different bacterial origin to GPCRs. However, both mPRs and PGMRC1 appear to function as oligomers, like GPCRs [80]. Moreover, a recent study by Karteris et al. shows human mPR α is rapidly internalized upon P4 stimulation and that the receptor is present in clathrin-coated pits [unpubl.obs].

b. Expression of recombinant putative progestin membrane receptors

The detection of recombinant progestin receptors on plasma membranes of eukaryotic cells by radioreceptor assay is frequently complicated by the presence of high background progestin binding. Greatest background binding to membrane preparations is frequently observed with progesterone, which is more lipophilic than the teleost progestins, 17,20 β DHP and 20 β -S, and other major sex steroids, estradiol-17 β and testosterone. This problem can be minimized when expressing recombinant progestin membrane receptors in eukaryotic cells by selecting cell lines with low background binding and by maximizing expression of the receptor. Plasma membranes prepared from CHO and HEK293 cells were found to have higher background progesterone binding than those prepared from COS-7 and MDA-MB-231 breast cancer cells using a filtration assay [unpubl. obs.]. MDA-MB-231 cells do not express nuclear progesterone receptors and therefore have been used for expressing recombinant mPR α in order to determine its steroid binding and signaling characteristics [122,150]. However, CHO cells have been used successfully for the stable expression of *Xenopus* mPR β and PGMRC1 [4,26]. Both the putative

membrane progesterin receptors, mPRs and PGRMC-1, are widely expressed in mammalian tissues and immortal mammalian cell lines (Fig. 6), including MDA-MB-231 cells, and may partially account for the high background progesterin binding in some of these cells. For example weak expression of mPR α protein and some specific progesterin binding can be seen in MDA-MB-231 cells transfected with an empty vector (Fig. 7C). This endogenous expression of putative progesterin membrane receptors in eukaryotic expression systems further complicates investigations of the characteristics of the recombinant receptor proteins. Fortunately, endogenous progesterin receptor expression in MB-MDA-231 cells is insufficient to mediate activation of G proteins and alterations in the activities of adenylyl cyclase and MAPkinase by progestins and these responses are only observed when a membrane progesterin receptor such as mPR α is overexpressed [122,150]. In the initial study expression of mPR α protein in MBA-MD-231 cells stably transfected with the seatrout mPR α cDNA was sufficient to transduce the progesterin signal intracellularly to alter second messenger pathways, but not to demonstrate a significant increase in specific progesterin binding [150]. Several strategies have been used to increase the expression of recombinant steroid membrane receptors. Stable transfection and continued selection over an extended period of up to 6 weeks has proven to be effective for increasing plasma membrane expression and progesterin binding of goldfish and zebrafish mPR α s [37,132]. However, for seatrout and human mPR α clones expressing high amounts of the receptor protein and progesterin membrane binding were further selected by the limiting dilution method. Twelve aliquots of the transfected cells were serially diluted in 96-well plates and selection was continued for one week, after which selection was continued for a second week with plates containing only one or two healthy clones. The cells were then transferred to a cell culture flask and cultured for 10-14 days with several changes of medium. The cells were subcultured and after 1-2 passages there was a sufficient number of cells for expression and functional analyses. The expression of the human mPR α protein and [3 H]-progesterone binding in plasma membranes prepared from ten separate clones obtained by this procedure are shown in figure 7A and 7B. Binding of [3 H]-progesterone to the membrane preparations varied among the clones and in general was correlated with mPR α protein expression. Four clones with high receptor binding were selected for continued selection and subculture. High receptor binding was retained after several passages (fig. 7 C) by which time there were enough cells for comprehensive characterization of the receptor. An alternative more rapid method of selecting cells with high cell surface expression of receptors is by cell sorting with a flow cytometer [14]. This method has been used successfully to enrich transfected cells expressing GPR30 on the cell surface [31]. Finally, transient transfection has been used successfully to express ovine mPR α and *Xenopus* mPR β [1,4] and may be adequate for limited characterization studies. The limiting dilution method has also been used successfully to obtain individual clones of CHO cells stably transfected (selection was maintained for two weeks) with PGMRC1 expressing sufficient amounts of the protein for [3 H]-progesterone binding studies [26].

c. Measurement of recombinant putative progesterin membrane receptors

Many hormone membrane receptors rapidly lose binding activity upon membrane disruption and homogenization unless precautions are taken to minimize degradation of the receptor protein. For the mPR α radioreceptor assay the entire procedure is conducted at 4C, a protease enzyme inhibitor cocktail is added in the homogenization and assay buffers, and the assay incubation period is shortened to 30 minutes which is sufficient for the binding to reach equilibrium. Progesterin tracer bound to the receptor is rapidly separated from free steroid (< 20 sec.) by retaining the membrane fraction on glass fiber filters using a filtration manifold attached to a vacuum pump in the cold room (~6C). Omission of the protease inhibitor cocktail from the buffers results in a 32 % loss of specific receptor binding (Fig.8). Progesterin binding to recombinant mPRs was not detected in recent study by Brosens and coworkers [47] using an incubation period of 1hr at room temperature. Our results show that a 1hr at 4C does not

cause a significant decline in receptor binding compared to the 30 minute incubation period, whereas conducting the incubation at room temperature for 1hr resulted in a 45-55 % decline in receptor binding even in the presence of protease enzyme inhibitors (Fig.7). Similar results were obtained for the progesterone membrane receptor on *Xenopus* oocytes; after one hr incubation at 20C, [³H]-progesterone binding had decreased to approximately 50% of that measured after incubation at 4C [50].

Concluding Remarks

As expected for a new emerging field, many aspects of progestin signaling through novel putative progestin receptors remain unresolved and surrounded by controversy. However, considerable progress has been made in our understanding of the characteristics and physiological functions of mPR α in the short period since its discovery in seatrout ovaries was reported in 2003 [150]. The progestin binding characteristics of recombinant mPR α s has recently been confirmed in eukaryotic expressions systems for six vertebrate species (seatrout, goldfish, zebrafish, *Xenopus*, sheep, human) by four different research groups [1,4,122,132]. Activation of signal transduction pathways by progestins in mPR α -transfected cells has now been demonstrated by four research groups for mPR α s of five vertebrate species [1,4,37, 122]. Moreover, impairment of progestin actions (binding, induction of signal transduction pathways, or a biological response) by prior treatment with siRNA, antisense oligonucleotides, or specific antibodies has been independently confirmed in four laboratories in different vertebrate and cell models [1,41,133,150]. In addition, the wildtype mPR α protein has been localized to the plasma membranes of a variety of cell types including seatrout, goldfish, zebrafish and *Xenopus* oocytes, fish sperm and human myocytes by investigators in five laboratories [4,37,41,127,150], although the protein is also present in intracellular compartments [41]. Finally, clear evidence for hormonal regulation of mPR α has been obtained by three research groups in different vertebrate models [41,133,150]. Taken together, the results of these studies strongly support the original suggestion [150] that mPR α functions as a progestin membrane receptor that is expressed on the plasma membrane and acts as an intermediary in progestin activation of intracellular signaling pathways. In marked contrast to the results of these research groups, Brosens and coworkers reported that recombinant mPR α s of several vertebrate species were not present on the plasma membranes of transfected cells, but were localized in the endoplasmic reticulum [47]. In addition, they did not detect any specific progesterone binding to the microsomal fractions of these mPR α -transfected cells under the radioreceptor assay conditions they employed [47]. The reasons these investigators failed to detect progesterone binding to recombinant mPR α are unclear, but are probably at least partly attributable to methodological problems, such as inappropriate radioreceptor assay incubation conditions (Fig.8), receptor binding measurement only in microsomal fractions [122], and the expression of some of the recombinant proteins with apparent molecular weights (28 kD) much lower than that of mPR α (~40kD) and the results obtained by other investigators. The findings obtained by other researchers were not directly evaluated by Brosens and coworkers because they did not examine [³H]-progesterone binding or the presence of the mPR α protein by Western blotting in plasma membrane preparations obtained by subcellular fractionation of stably transfected cells [47], so their conclusions from this study are enigmatic. It should be noted, however, definitive evidence that mPR α is membrane progestin receptor has not been obtained to date. The demonstration of a plausible progestin binding site on mPR α by mutational analysis will be required for direct evidence that it functions as a progestin receptor. Moreover, an involvement of intracellular mPR α pools in activating signal transduction pathways cannot be excluded, since steroids can readily diffuse into the intracellular compartments. In addition, practically no information is currently available on the regulation of cell surface expression, trafficking, and endocytosis of mPR α , the role of dimerization in receptor function, its association with other proteins, and the mechanisms of

cross-talk with nPRs. At the current rate of progress it is anticipated that new information on at least some of these processes will be obtained in the next few years.

Currently, even less is known about the progestin membrane receptor characteristics and functions of PGMRC1. The diverse range of ligands and proteins that can interact with the protein as well as the bewildering array of processes it is associated with, including physiological, developmental and toxicological ones, suggests PGMRC1 has widespread and important functions in the cell. However, in view of its widespread possible functions and interactions, it is unclear whether PGMRC1 has a distinct role as a specific membrane progestin receptor alone, or when coupled to another protein such as PAIRB1. A clear demonstration that progesterone activates signal transduction pathways via a plausible mechanism through PGMRC1 or this protein complex in several cell models, such as that obtained for mPR α through an inhibitory G protein, will be required to establish it as a functional progestin membrane receptor. Experiments demonstrating that down-regulation of PGMRC1 expression after treatment with siRNAs or antisense oligonucleotides is associated with decreases in [³H]-progesterone binding and progesterone signaling are also necessary but have not been conducted. Moreover, the ability of the recombinant protein produced in a prokaryotic expression system to bind [³H]-progesterone should be re-examined in view of the negative findings of a previous study [67]. In addition, mutation experiments, like those proposed for mPR α , will be required to identify the progesterone binding site. Finally, the potential role of PGMRC1 as an adaptor protein warrants further investigation.

A notable feature of both mPR α and PGMRC1 is their widespread tissue distribution. The finding that these putative progestin membrane receptors are co-expressed in all the eukaryotic expression systems examined to date (Fig.6), further complicates investigations of their individual characteristics. Therefore, future studies need to take into account the presence of the other putative progestin membrane receptor when examining the characteristics of mPR α or PGMRC1.

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Figure 1. DEAE chromatography of solubilized seatrout ovarian membranes with step wise elution of protein with NaCl. Three fractions (I,II, III) were collected and PAGE was performed on aliquots from each fraction and silver stained. Reproduced from Zhu et al. [150], with permission.

Figure 2.

Competition curves of steroid binding to plasma membranes of MDA-MB-231 (PR-) cells stably transfected with (A) human mPRα, (B) seatrout mPRα, (C) zebrafish mPRα, expressed as a percentage of maximum [³H]progesterin binding (A: [³H]P4; B: [³H]20β-S; C: [³H]17α, 20β-P). P4, progesterone; 20β-S, 17,20β,21-trihydroxy-4-pregnen-3-one; 17α,20β-P, 17,20β-dihydroxy-4-pregnen-3-one; RU486, mifepristone; R5020, promegestone; cort, cortisol; E2, estradiol-17β; T, testosterone. (A and B reproduced from Thomas et al., 2007 [122], C reproduced from Hanna et al., 2005 [37] with permission).

Figure 3.

Co-immunoprecipitation of mPR α coupled to G protein α subunits in spotted seatrout oocyte membranes with specific inhibitory G protein (G $_{i1,2}$, G $_o$) antibodies followed by immunodetection of seatrout mPR α by Western blot analysis. Membranes (10 μ g) were incubated for 45 min. with vehicle or 290 nM 20 β -S, solubilized and then incubated overnight with 5 μ l of G $\alpha_{i1,2}$ or G α_o antibody or nonimmune rabbit serum (niR) and 50 μ l of Protein A/G Plus-agarose beads. Immunoprecipitated seatrout mPR α protein was detected using the monoclonal PR10-1 antibody described in Zhu et al. (2003). Representative immunoblot is shown. There was a strong and specific association of G $\alpha_{i1,2}$ with seatrout mPR α , whereas the mPR α protein was not detected in the G α_o or nonimmune rabbit serum immunoprecipitated samples. The amount of seatrout mPR α associated with G $\alpha_{i1,2}$ was decreased after treatment with 20 β -S (reproduced from Pace, 2005 [81] with permission).

Figure 4.

Down regulation of coactivator SRC2 mRNA expression in human pregnant myometrial cells treated with 100 nM progesterone-bovine serum albumen conjugate (P4-BSA) for 3hrs. * $P < 0.05$ compared to no supplement (NS) controls, $N=4$. SRC2 protein expression (insert) was also decreased after 16hr treatment (reproduced from Karteris et al., 2006, with permission). CBP: cAMP response element binding protein coactivator. SRC: steroid receptor (PR) coactivator (reproduced from Karteris et al., 2006 [41] with permission).

mPR α

PGRMC1

Figure 5.

Summary of the signal transduction pathways activated by progestins through mPR α (1-8) and PGRMC1 (8). mPR α pathways 1-4, 6, 7 are mediated through activation of inhibitory G proteins (G_i), mPR α pathway 5 is mediated through an olfactory G protein (G_{olf}). Pathway 1. Activation of Erk (Erk1, Erk2): Atlantic croaker oocytes during final maturation, probably through mPR α [82] and in MDA-MB-231 cells transfected with seatrout mPR α [150] and zebrafish mPR α and mPR β [37]. Pathway 2: Activation of mitogen activated kinase p38: PTX-sensitive, via the mPRs in human myocytes resulting in phosphorylation of myosin light chain [41]. Pathway 3: Akt activation: Atlantic croaker oocytes during final maturation, probably through mPR α , resulting in upregulation of phosphodiesterase activity [82]. Pathway 4: Decrease

cAMP levels: PTX-sensitive, in spotted seatrout oocytes during final maturation through mPR α [83, Fig. 2], in MDA-MB-231 cells transfected with seatrout mPR α or human mPR α [122, 150], and in human myocytes via mPR α and mPR β [41], in Jurkat cells via mPR α [21]. Pathway 5: Increase cAMP levels: in Atlantic croaker sperm through mPR α [135]. Pathway 6: Potentiation of PR-B transactivation: through mPR α - and mPR β -mediated pathways in human myocytes [41]. Pathway 7: Down-regulation SRC2 coactivator expression: through mPR α in human myocytes [41]. Pathway 8: Increase intracellular free calcium concentrations: from internal calcium stores in CHO cells transfected with ovine mPR α [1] and in CHO cells transfected with PGMRC1 [26]. PM: plasma membrane, NE: nuclear envelope, ER: endoplasmic reticulum.

Figure 6. Detection of mPR α and PGMRC1 mRNAs in various cell lines after 35 cycles of RT-PCR. MDA: MDA-MB-231, SKBR: SKBR3, MYO: human myometrial cells after several passages, HEK: HEK293, RT-: lacking reverse transcriptase for actin controls, showing no DNA contamination.

Figure 7.

Selection of membrane progesterin receptor-transfected cells for receptor characterization and signal transduction studies. Binding of [3 H]-P4 to plasma membranes of MDA-MB-231 cells stably transfected with human mPR α . A and B: Binding to ten subclones (a1-a10), obtained by limiting dilution, was assessed after three weeks selection. High [3 H]-P4 binding was associated with greater mPR α protein expression in the membrane fractions (insert) as assessed by Western blot analysis. C: [3 H]-P4 binding increased upon further selection for two weeks of the clones which had the highest binding in A&B (a1,a3,a5,a10). The receptor binding assay was conducted as described in Thomas et al., 2007. * $P < 0.05$ compared to untransfected control cells (231-ve), Student's paired t test, $N = 4$.

Figure 8.

Loss of [3 H]-P4 binding to plasma membranes prepared from MDA-MB-231 cells stably transfected with human mPR α during incubation at room temperature for 1hr (RT-1hr) or in the absence of protease inhibitors (-prot.inhib). The receptor binding assay was conducted as described in Thomas et al., 2007 [122] with modifications from the normal 30 min incubation procedure at 4C in the presence of protease inhibitors as indicated. Significant differences identified with a multiple range test, Fisher PLSD, are indicated with different letters ($P < 0.05$), $N=4$.