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Steroidogenic activities in MA-10 Leydig cells are differentially altered by cAMP and Müllerian inhibiting substance

Alexander M. Trbovich[a], Nina Martinelle[a,b], Francis H. O'Neill[a], Elliot J. Pearson[a], Patricia K. Donahoe[a], Patrick M. Sluss[c], Jose Teixeira[a,*]

[a] *Pediatric Surgical Research Laboratories/WRN 1024, Massachusetts General Hospital and Harvard Medical School, 32 Fruit Street, Boston, MA, USA*
[b] *Pediatric Endocrinology Unit, Department of Woman and Child Health, Astrid Lindgren Children's Hospital, Karolinska Institute, Stockholm, Sweden*
[c] *Reproductive Endocrine Sciences Center, Massachusetts General Hospital and Harvard Medical School, Boston, MA, USA*

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Abstract

In addition to causing Müllerian duct regression in fetal males, Müllerian inhibiting substance (MIS) inhibits the expression of the bifunctional cytochrome P450, C17 hydroxylase/C_{17-20} lyase (Cyp17), the enzyme that catalyzes the committed step in sex steroid synthesis. To investigate the paracrine effects of MIS on steroidogenic activity, we have performed assays with microsomes from mouse MA-10 Leydig cells. With microsomes from untreated MA-10 cells, progesterone was largely metabolized by 5α-reductase and subsequently converted by 3-keto steroid reductases to allopregnanolone and epiallopregnanolone. Addition of cAMP to the cells shifted microsomal steroid production to the Cyp17 product androstenedione and its 5α,3β-reduced form, epiandrosterone. Microsomes from MIS-treated cells were less active with the progesterone substrate than those of untreated cells but co-treatment of the cells with both MIS and cAMP mitigated the cAMP-induced shift of the microsomes to androstenedione production. Quantitative analyses of steroid production by Cyp17 showed that cAMP decreased the amount of 17-hydroxyprogesterone produced relative to the androstenedione, suggesting that cAMP signaling lowers the efficiency of the Cyp17 hydroxylase activity or else increases the efficiency of its lyase activity. Addition of MIS to the cAMP-treated cells partially reversed this effect, as well. These results indicate that cAMP induces MA-10 cells to switch from producing 5α-reduced progesterone metabolites to producing androstenedione and its metabolites by increasing Cyp17 expression and its relative lyase activity, both of which are inhibited by MIS.

Keywords: Müllerian inhibiting substance; Cyp17; Leydig cells; 5α-Reductase

1. Introduction

Müllerian inhibiting substance (MIS, also known as anti-Müllerian hormone or AMH [1]) is a glycoprotein hormone member of the transforming growth factor-β (TGFβ family of cytokines that is produced by Sertoli cells shortly after commitment of the bipotential gonad to testis differentiation and is required for regression of the Müllerian ducts (MD) in the male fetus (reviewed in [2]). The absence of MIS in females or its downstream signaling in males with persistent Müllerian duct syndrome, allows the MDs to develop into the internal female reproductive tract: the Fallopian tubes, uterus, and the upper portion of the vagina. MIS continues to be expressed in males at a relatively high level until puberty, well after MD regression has occurred. Coincident with puberty, MIS expression in males decreases to a lower level, which is similar to that observed in females, who begin expressing MIS during the neonatal period [3]. Regulation of steroidogenesis [4–6], follicular development [7,8], and tumor sup-

Abbreviations: MIS, Müllerian inhibiting substance; MD, Müllerian duct; Cyp17, cytochrome P450 C17 hydroxylase/C17-20 lyase; A, androstenedione; T, testosterone; P, progesterone; 17-OHP, 17-hydroxyprogesterone; 5α-P, 5α-pregnan-3,20-dione; 5α,3β-P, 5α-pregnan-3β-ol-20-one, epiallopregnanolone; 5α-A, 5α-androstan-317-dione; 5α,3β-A, 5α-androstan-3β-ol-17-one, epiandrosterone; HSD, hydroxysteroid dehydrogenase; KSR, ketosteroid reductase

* Corresponding author. Tel.: +1 617 724 1616; fax: +1 617 724 1627.
E-mail address: teixeira@helix.mgh.harvard.edu (J. Teixeira).

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pression [9–11] have all been ascribed to the physiological significance of MIS expression in the adult. Additionally, recent evidence suggests that natural and synthetic estrogens may be inhibiting folliculogenesis by increasing MIS expression in the ovary [12–14]. These putative postnatal roles for MIS expression have greatly expanded the interest in MIS signaling.

Male mice that are deficient in MIS signaling either from deletion of the MIS ligand or its requisite type II receptor have retained MD-derived structures and have hyperplasia of the gonadal testosterone-producing Leydig cells [15]. Females suffer from increased follicle recruitment and subsequent premature ovarian senescence [8]. Male mice overexpressing MIS are undervirilized because of low testosterone levels that result from the relative paucity of Leydig cells [16,17] as well as MIS-mediated suppression of testosterone synthesis by these cells, which has been shown both in vitro [18,19] and in vivo [5]. The physiological role for the paracrine regulation of steroidogenesis by MIS has yet to be determined but abnormalities in reproductive tissues because of perturbed MIS signaling suggest that MIS is required for normal reproductive function.

We are using MA-10 cells to study the paracrine effects of MIS on steroidogenesis. MA-10 cells express the MIS type II receptor (MISRII) [4], which is required for MIS signal transduction [4,15] and they respond to LH and cAMP by increasing steroid hormone production [20], including low but measurable amounts of testosterone [6]. The major metabolites of MA-10 cells with and without induction by hCG have been described [21] but their steroidogenic responses to MIS have not been thoroughly investigated.

The cytochrome P450 C17 hydroxylase/C17-20 lyase (Cyp17) is a bifunctional microsomal enzyme that catalyzes the hydroxylation of the carbon 17 of the C21 steroids, pregnenolone and progesterone, and performs the committed step in androgen biosynthesis, the cleavage of the C17–C20 bond, which converts these C21 steroids to the C19 steroids, dehydroepiandrosterone and androstenedione, respectively [22]. Gonadal Cyp17 is found in the Leydig cells of the testis and the theca cells of the ovary, the major steroid producing cells of the gonads. Its expression is induced by LH binding to its G protein-coupled receptor, which activates adenylyl cyclase and subsequently, the cAMP-dependent protein kinase A (PKA). Following production of androstenedione, 17-ketosteroid reductase (KSR) converts the 17-keto group to a hydroxyl, generating testosterone. Dihydrotestosterone (DHT) is a potent androgen that is made from testosterone by the activity of 5α-reductase, which exists as two isozymes encoded by two different genes [23].

We have shown that, in MA-10 cells, MIS inhibits the expression of the Cyp17 at the transcriptional level [4] by blocking the PKA signaling pathway [6]. Additionally, in both in vivo and in vitro assays for 17-hydroxyprogesterone (17-OHP) and testosterone, MIS appears to inhibit the production of testosterone more effectively than it does 17-OHP [5,6]. Whereas with MIS treatment the testosterone concentration, either in serum or in conditioned media, was routinely 1/10th that observed without MIS, the 17-OHP concentrations, although significant, were only 10–20% lower with MIS. We speculated that, in addition to its inhibition of Cyp17 expression, MIS might be affecting the Cyp17 lyase activity. Other possibilities are that this difference could be the result of inhibition of ketosteroid reductase expression or activity by MIS or that MIS might be inducing the expression of another enzyme that converts testosterone to another metabolite with the resultant lowering of its concentration.

Here, we report our studies comparing and contrasting microsomal steroidogenesis and the differential response of MA-10 cells to cAMP and MIS. We show that cAMP causes the cells to switch from producing progesterone metabolites to producing androstenedione, that cAMP increases the Cyp17 lyase activity relative to its hydroxylase activity, and that MIS partially reverses both these effects.

2. Material and methods

2.1. Chemicals and reagents

Radionucleotides were purchased from Amersham Pharmacia Biotech, Piscataway, NJ, or Perkin-Elmer, Boston, MA. Waymouth's MB 752/1 medium, gentamicin, and horse serum were purchased from Invitrogen Life Technologies Inc., Carlsbad, CA. Steroids were from Steraloids, Newport, RI or Sigma, St. Louis, MO. All other chemicals were obtained from Sigma or Fisher Scientific, Pittsburgh, PA unless otherwise noted. MK386 was a kind gift from Dr. Barry Gertz (Merck Research Laboratories, Piscataway, NJ). Mouse 5α-reductase cDNAs [24] were from Dr. Mahendroo (UT Southwestern, Dallas, TX). MA-10 cells were provided by Dr. Mario Ascoli, University of Iowa, Iowa City, IA [20]. The 5α-reductase type I antibody was from Santa Cruz Biotechnology, Santa Cruz, CA. Recombinant human MIS was expressed and purified as described earlier [25].

2.2. Preparation of microsomes

Mouse Leydig MA-10 cells were cloned and cultured as previously described [6] and plated at 2×10^6 cells per 100 mm dish on day 0. Culture medium was changed on day 2 prior to treatment. There were four experimental groups: no treatment (NT), 50 μM 8Br-cAMP, 50 μM 8Br-cAMP with 35 nM MIS (MIS was added 30 min prior to cAMP treatment), and 35 nM MIS alone. Cells were incubated overnight at 37 °C, and were collected at day 3 or 4 for TLC or HPLC, respectively. To isolate microsomes, cells were resuspended in 5 ml of microsomal buffer (10 mM Tris pH 7.4, 1 mM EDTA, 0.3 M sucrose) and then dounce homogenized with a tight pestle. The suspension was then centrifuged at $2000 \times g$ for 20 min at 4 °C and the supernatant was collected for a second spin ($10^5 \times g$ for 1 h at 4 °C). The microsomal pellet was then resuspended by gentle douncing in 1/5 of the original buffer

volume. Testes were harvested from 150 to 175 g rats 24 h after they were injected with 100 IU LH or with LH and 1 mg MIS i.p. [5] for preparation of testicular microsomes as above for cells except that the first centrifugation was performed at 27,000 × *g*. All animal protocols were approved by the Institutional Animal Care and Use Committee. Protein content was measured by the Bradford method [26].

2.3. Steroid assays

In a given experiment, 10^5 cpm of ^{3}H- or ^{14}C-labeled steroids were diluted with unlabeled substrate for use as substrates and mixed with 100 μg protein microsomes in a total volume of 200 μl of reaction buffer (200 mM Hepes pH 7.4, 0.2 mM EDTA). Final concentration of steroid substrates was 10 nM–2 μM for the MA-10 cells and 7 μM for rat testicular microsomes. Except where stated, NADPH was also added at a final concentration of 1 mM to the reaction mix. MK386 was used at a final concentration of 100 nM. Microsomal assays were performed for 30 min and the reaction was stopped by simultaneous addition of ethanol (500 μl) and 0.1N HCl (20 μl). Steroids were extracted with diethyl ether and dried. The assays were repeated at least twice.

2.4. Thin layer chromatography

Steroids were spotted on 250 μm layer silica plates with a fluorescence indicator (Whatman, Germany). Unlabeled standards were separated in parallel experiments to identify the tritiated metabolites by migration distance. The silica plate was run for approximately 2 h in a glass-sealed chamber previously equilibrated for approximately 90 min with chloroform/ethylacetate (3:1) [27]. When the solvent front was 2 cm below the top of the plate, it was removed from the chamber and air-dried. Cold steroids were visualized with short-wave ultraviolet light or by incubating the TLC plate in a sealed chamber with iodine pellets. With ^{3}H-steroids, En^{3}Hance Spray (Perkin-Elmer) was applied to the silica plate before exposure to film. TLC experiments shown are representative of at least three separate experiments.

2.5. High-performance liquid chromatography

Resuspended steroids were centrifuged at $10^4 \times g$ for 5 min. The supernatants were collected and injected onto a 5 μm, 4.6 × 150 mm C_{18} Symmetry column (Waters, Bedford, MA), which was developed using a step gradient generated by Water 510 HPLC pumps. The solvent system was 50% methanol for the fractions 1–35, and 70% methanol for the fractions 36–70. The flow rate was 1 ml/min and the fraction size was 2 ml. All 70 fractions were collected and the amount of radioactivity was determined by scintillation counting. The cpm for each fraction was corrected for total recovery before calculating the mass of the steroid peaks based on specific activity. For the experiments where the unknown steroids were isolated, 10 μl of each fraction was counted to determine the exact position of the peak. The remainder of the fraction was dried by N_2 then reconstituted in 200 μl of methanol for the HPLC experiments and 20 μl of methanol for the TLC experiments. The cpm for 17-OHP, androstenedione, and testosterone were combined to determine 17-hydroxylase activity and the cpm for androstenedione and testosterone were combined to determine the 17–20 lyase activity. The mean cpm from two replicate experiments was used for calculations and graphical analyses performed with Prism software (GraphPad, San Diego, CA). Significant differences between the ratios of products were determined by ANOVA using Bonferroni's multiple comparison test of the aggregate data for each activity.

2.6. Northern and Western analyses

MA-10 cells were treated as above with cAMP and MIS overnight before total RNA was isolated using Trizol (Invitrogen) and quantitated by absorbance at A_{260}. RNA (10 μg) samples were denatured with dimethylsulfoxide and glyoxal at 65 °C, separated in a 1.5% agarose gel, blotted overnight onto nylon membranes, and UV-cross linked. Blots were prehybridized with 100 μg/ml sonicated salmon sperm DNA in 50% formamide hybridization solution and hybridized overnight at 65 °C with 2×10^6 cpm/ml ^{32}P-labeled antisense riboprobes against mouse 5α-reductase types 1 and 2 [24]. Blots were washed at 65 °C with 0.1× SSC (1× SSC = 150 mM sodium chloride and 15 mM sodium citrate)–0.1% SDS and exposed to radiographic film with intensifying screens at −70 °C. The Western blot for 5α-reductase type I protein expression was performed using standard techniques. Approximately 15 μg of microsomal protein was electrophoresed on a 4–12% Novex gel (Invitrogen) with MOPS buffer, transferred to nitrocellulose and probed with antibody according to the supplier's instructions. Relative molecular weight was determined with MagicMark (Invitrogen) protein ladder. Both northern and Western blots were repeated at least once with similar results.

3. Results

3.1. MIS and cAMP alter the microsomal activities of MA-10 cells on progesterone

In order to test the hypothesis that, in addition to inhibiting the expression of Cyp17, MIS might also be affecting other enzymatic reactions involved in androgen biosynthesis and or progesterone metabolism, we prepared microsomes from MA-10 cells either untreated or treated variously with 50 μM 8Br-cAMP (cAMP), 35 nM MIS, or both. MA-10 cells are a mature mouse Leydig cell line that we and others have used to recapitulate the effects of exogenous treatments on steroidogenesis [20,21]. We have previously shown that there is a several-fold induction of Cyp17 mRNA in MA-

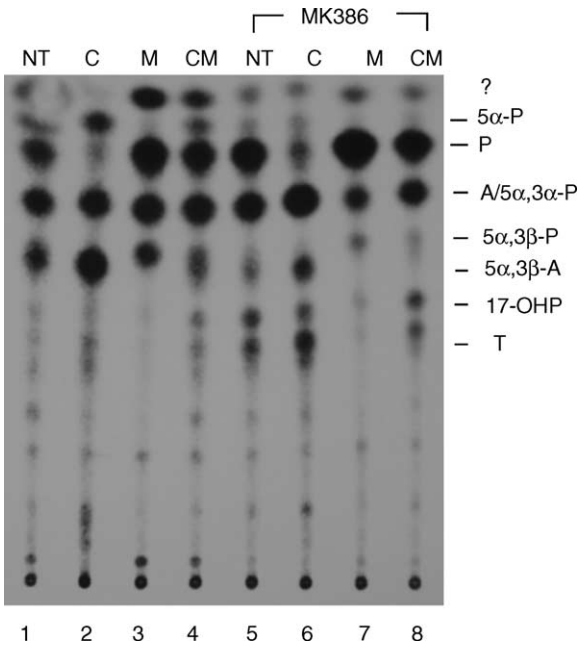

Fig. 1. Thin layer chromatography of progesterone metabolites from assays with MA-10 microsomes. MA-10 cells were treated overnight as indicated (NT, no treatment; C, 50 μM 8Br-cAMP; M, 35 nM MIS; CM, both cAMP and MIS). Microsomes from the cells were incubated with 200 nM ^{3}H-progesterone substrate as described in Section 2 with or without MK386, a 5α-reductase type 1-specific inhibitor. After completion of the assay, the steroids were extracted and separated by TLC in chloroform:ethyl acetate, 3:1. The plate was sprayed with En^{3}Hance and exposed to X-ray film for approximately 1 week at −70 °C. The metabolites were identified by their migration distance relative to standards (indicated on the right).

10 cells treated with 50 μM 8Br-cAMP within 4 h that is inhibited by co-treatment with 35 nM MIS, the concentration normally required for complete Müllerian duct regression in an organ culture assay [6]. Fig. 1 shows the qualitative results of a representative TLC experiment performed with 200 nM progesterone substrate concentration for 30 min. The reduced progesterone, allopregnanolone, (5α,3α-P) was the most abundant product of microsomes prepared from untreated cells (lane 1) with smaller amounts of steroids that co-migrate with epiallopregnanolone (5α,3β-P) also observed. Androstenedione and 5α,3α-P migrate the same distance in the chromatography system used, therefore from these results we cannot preclude the possibility that untreated cells also produce androstenedione. If the microsomes were incubated with MK386, a 5α-reductase type 1-specific inhibitor (lane 5), more progesterone substrate remains unconverted and the amount of 5α,3β-P is greatly reduced. In contrast, addition of cAMP to the cells (lane 2) shifted their steroid production so that androstenedione and its 5α,3β-reduced form epiandrosterone (5α,3β-A) were the major products of the microsomes. Additionally, all of the progesterone substrate was converted with the microsomes from cAMP-stimulated cells suggesting that kinetics of Cyp17 are much more efficient than those of 5α-reductase with progesterone under these conditions. Conversion of androstenedione to 5α,3β-A was greatly diminished by the addition of MK386 (lane 6).

Treatment of the cells with MIS (lane 3) resulted in an increased level of unused progesterone substrate but as with the untreated microsomes, the conversion of progesterone observed was to 5α,3α-P and to a lesser extent 5α,3β-P, the production of both of which were inhibited by MK386 (lane 7). The effect of treating cells with both MIS and cAMP (lane 4), resulted in more progesterone substrate remaining unconverted than with microsomes from cells treated with cAMP alone and the production of 5α,3β-A was also inhibited, suggesting that the major product of these microsomes is 5α,3α-P. However, addition of MK386 shows that microsomes from cells treated with both MIS and cAMP are likely to produce a combination of androstenedione and 5α,3α-P since (lane 8). In all the above studied reactions, the amount of 17-OHP observed was trivial indicating that very little is released from the Cyp17 enzyme before conversion to androstenedione. If either microsomes or NADPH was not included in the reactions, no conversion of progesterone was observed indicating that all the observed metabolites were enzymatically generated by an NADPH cofactor-dependent process (data not shown).

3.2. MA-cells show very little microsomal activity with downstream substrates

Microsomal assays were also performed with 17-OHP, androstenedione, and testosterone at 200 nM substrate concentrations to determine whether their conversion to other steroids might be affected by MIS or cAMP (Fig. 2). Microsomes from untreated cells showed very little activity with the 17-OHP substrate (Panel A, lane 1) but did convert some 17-OHP to androstenedione. Using microsomes from cAMP-stimulated cells (Panel A, lane 2) showed a marginal increase in androstenedione production over that of untreated microsomes with the 17-OHP substrate in contrast to the amount of androstenedione produced with progesterone as the substrate (Fig. 1, lane 2). As has been previously reported [28,29], 17-OHP is a relatively poor substrate for conversion to androstenedione by the lyase activity of Cyp17 when compared to progesterone. However, as with progesterone, addition of MIS to the cells strongly repressed the lyase activity, both in the absence (Panel A, lane 3) and in the presence of cAMP (Panel A, lane 4). The major metabolite of assays using microsomes from untreated cells or from cells treated with MIS and with androstenedione as the substrate is 5α,3β-A (Panel B, lanes 5 and 7). Microsomes from cells treated with cAMP were less able to reduce the androstenedione substrate (Panel B, lanes 6 and 8). As was observed with the progesterone substrate (Fig. 1), very little, if any, testosterone was produced by the microsomes incubated with androstenedione. Assays with microsomes from any of the cells with testosterone as the substrate showed negligible activity (Panel C) suggesting that in this microsomal assay system testosterone is a poor substrate for 5α-reductase type 1.

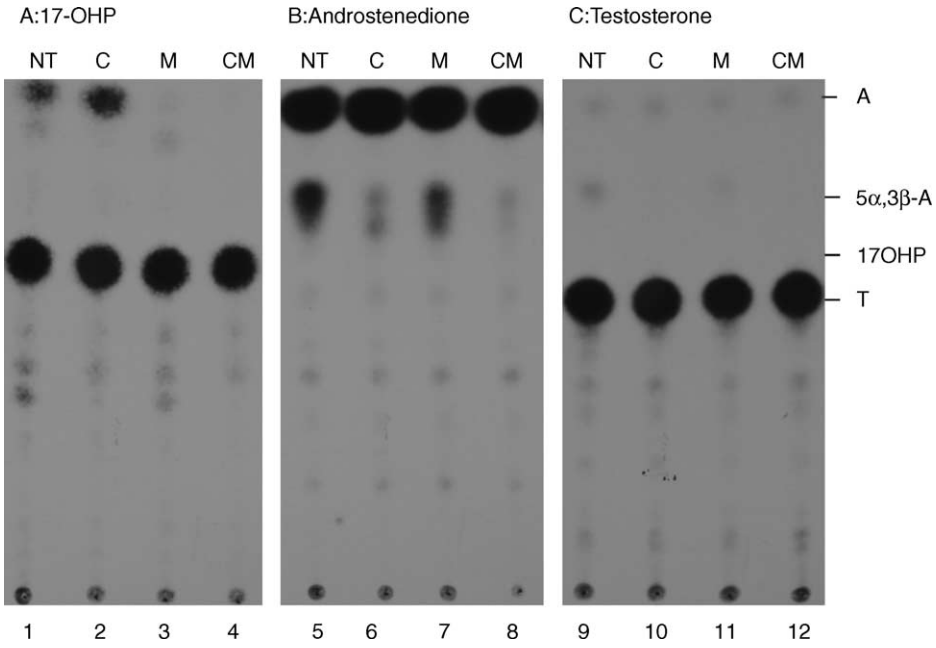

Fig. 2. TLC of androgen pathway substrates incubated with MA-10 microsomes. MA-10 cells were treated overnight as indicated (NT, no treatment; C, 50 μM 8Br-cAMP; M, 35 nM MIS; CM, both cAMP and MIS). Microsomes from the cells were incubated with 200 nM. Panel A: 17-hydroxyprogesterone, Panel B: androstenedione, or Panel C: testosterone as a substrate. Migration of standards is indicated on the right.

3.3. Neither cAMP nor MIS affect 5α-reductase 1 mRNA expression

In order to rule out the possibility that cAMP or MIS might have a confounding affect on steroidogenesis by regulating the expression of 5α-reductase type 1, we harvested total RNA from MA-10 cells after overnight treatment with cAMP and/or MIS for northern analysis (Fig. 3A). Neither cAMP nor MIS had any apparent effect on 5α-reductase type 1 mRNA expression. We also examined whether 5α-reductase type 1 protein expression was affected by the treatments using Western analysis but, as with the mRNA, did not observe any changes in the protein levels (Fig. 3B). The possibility that 5α-reductase type 2, which is normally not expressed in Leydig or MA-10 cells [30] might be regulated by cAMP or MIS was also examined but 5α-reductase type 2 mRNA expression was not detected (data not shown).

3.4. MIS and cAMP have opposing effects on progesterone metabolism.

We have shown, qualitatively, in Fig. 1 that cAMP and MIS alter the steroidogenic activities of MA-10 Leydig cells but determining any differential effect MIS might have on steroidogenesis, in general, and on the ratio of Cyp17 hydroxylase to lyase activities, in particular, required quantitative analyses using HPLC. Therefore, we first assessed whether the results from HPLC analyses reflected the results we had observed by TLC. Fig. 4 shows a representative experiment performed with 200 nM progesterone substrate concentration and MA-10 microsomes to ensure that the HPLC could reproduce the results we had observed by TLC. In Panel A, microsomes from untreated MA-10 cells convert the bulk the progesterone substrate to 5α-reduced progesterone metabo-

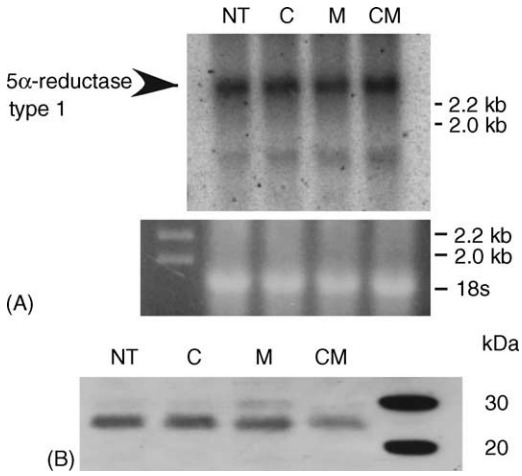

Fig. 3. Analysis of 5α-reductase type 1 expression in MA-10 cells. (Panel A) Total RNA from MA-10 cells was collected after overnight treatment of the cells as indicated. The RNA was denatured, separated by gel electrophoresis and blotted to a nylon membrane. The blot was cross-linked, probed with ^{32}P-labeled riboprobe to mouse 5α-reductase type 1, and exposed to X-ray film overnight at −70 °C with intensifying screens. Ethidium bromide stained RNA is shown to control for loading below the blot and migration of molecular weight standards are shown on the right. (Panel B) Microsomal 5α-reductase type 1 protein expression after overnight treatments as indicated was analyzed by Western blot. Approximately, 15 μg microsomes were subjected to PAGE, transferred to nitrocellulose and probed with a mouse 5α-reductase type 1-specific antibody.

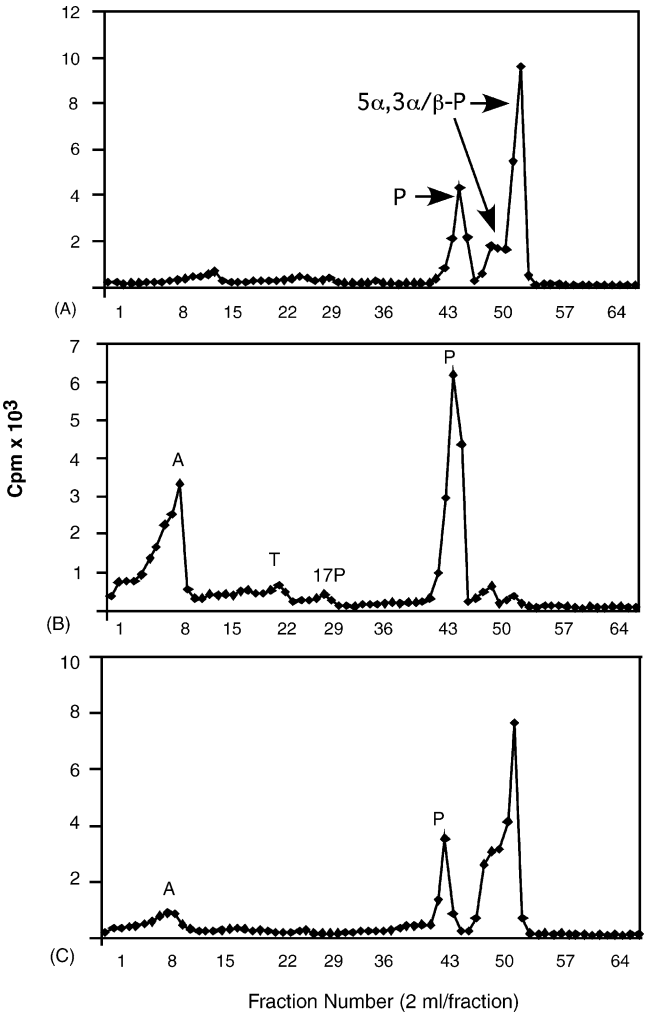

Fig. 4. Quantitative analysis of steroidogenesis in MA-10 cells. MA-10 cells were either untreated (Panel A), treated with 50 μM 8Br-cAMP (Panel B), or both (Panel C). Microsomes were harvested from the cells and incubated with 200 nM ^{3}H-progesterone (P) and 1 mM NADPH. At the end of the incubation, steroids were extracted, resolved by HPLC and counted as described in Section 2. The cpm for each HPLC fraction were normalized to the total cpm and plotted. The elution fraction of tritiated standards are shown with P, progesterone; A, androstenedione; T, testosterone; 17-OHP, 17-hydroxyprogesterone. 5α,3α/β-P are known metabolites of progesterone [21].

lites. The 5α-reduced peaks were collected and applied to a TLC plate and confirmed as allopregnanolone and epiallopregnanolone (data not shown). Additionally, if the collected fractions were used as a substrate in the microsomal reaction, no conversion was observed (data not shown). Because we were unable to collect the peaks separately, we could not assign the peaks to either 3α or 3β-reduced allopregnanolone or epiallopregnanolone products. However, since 5α,3α-P was the major product by TLC, we suspect that the major peak from the HPLC profile is also 5α,3α-P. In Panel B, androstenedione was the major product of microsomes prepared from cells that were stimulated with cAMP. As with the TLC results, relatively little 5α-reduced progesterone was produced compared to the untreated cells. Addition of MIS to the cAMP-stimulated cells greatly decreased the ability of the microsomes to produce androstenedione but not 5α-reduced progesterone (Panel C). Again, as with the TLC results, in all the reactions the amount of 17-OHP observed was trivial indicating that very little is released from the Cyp17 enzyme before conversion to androstenedione. Addition of MIS alone showed very little conversion of the progesterone above background (data not shown). If either microsomes or NADPH was not included in the reactions, no conversion of progesterone was observed indicating that all the observed metabolites were enzymatically generated by an NADPH cofactor-dependent process (data not shown). We therefore concluded that HPLC reflected the results we had observed by TLC and was a suitable mechanism by which to measure the effect of MIS on the Cyp17 hydroxylase activity relative to its lyase activity.

We also wanted to ensure that in the assays we were performing, the reactions were going to completion at 75 min in order to measure end products. We had previously observed that the progesterone substrate is depleted by 30 min in cAMP-stimulated cells but were unsure of the unstimulated cells. A time course with 200 nM progesterone substrate and with microsomes from untreated cells is shown in Fig. 5. By 75 min, nearly all of the progesterone substrate has been largely converted to the 5α-reduced products but with some of the Cyp17 products, as well.

3.5. MIS increases the production of 17-OHP by Cyp17 relative to androstenedione

MIS appears to inhibit the Cyp17 lyase activity more effectively than its hydroxylase activity [5], which we hypothesized was due to a differential effect of MIS on the efficiency of the two reactions performed by this bifunctional enzyme. We tested this hypothesis by performing microsomal assays with radiolabeled progesterone substrate. Fig. 6 shows the results of our studies plotted as the velocities of the reactions

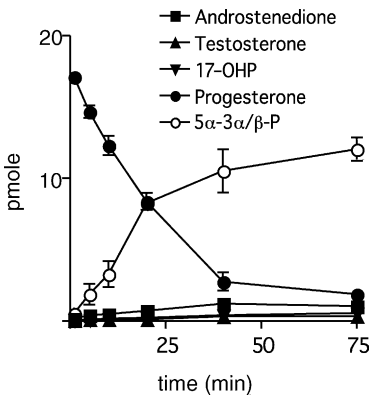

Fig. 5. Time course of microsomal activities. Microsomal assays were performed in duplicate with ^{3}H-progesterone for the indicated times and the steroids separated by HPLC. The cpm from each of the resultant peaks were measured, averaged from two replicate experiments and plotted. Error bars indicate the range of values.

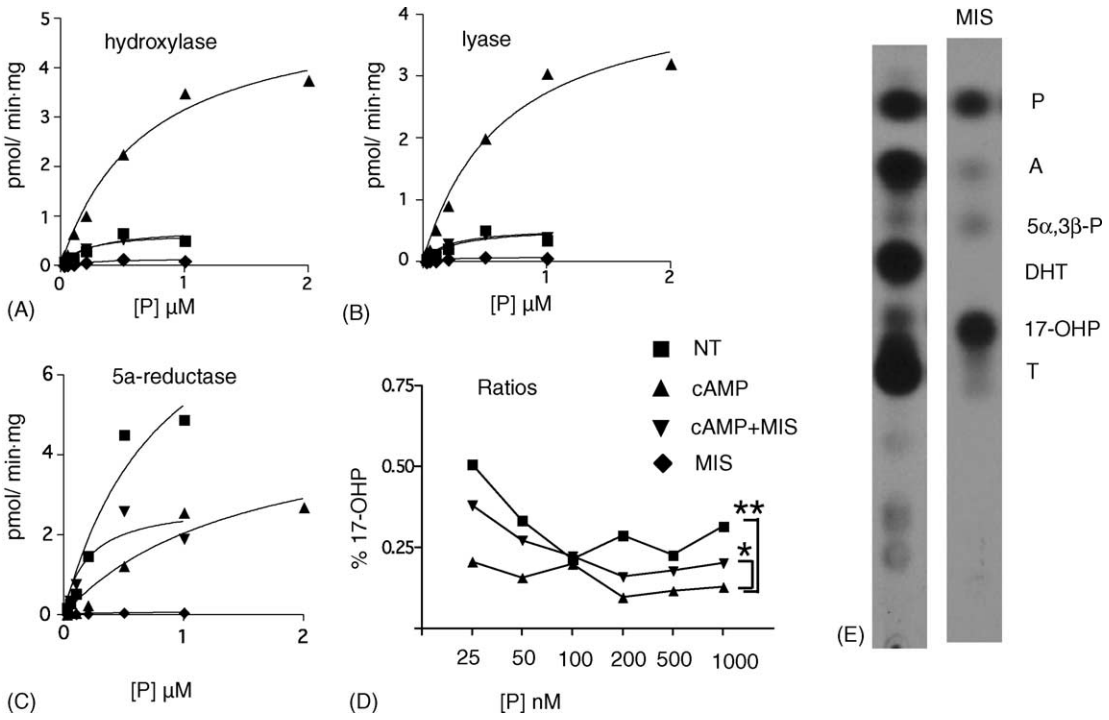

Fig. 6. Cyp17 activities in response to cAMP and MIS. Assays were performed in duplicate with microsomes harvested from MA-10 cells as in Fig. 1 with NADPH and increasing concentrations of ^{3}H-progesterone substrate for 75 min. The average velocity of Cyp17 hydroxylase activities (Panel A) for each concentration of progesterone was determined by combining the resultant amounts of 17-hydroxyprogesterone, androstenedione, testosterone produced and plotted. The average velocity of the Cyp17 lyase activities (Panel B) was determined by combining the amounts of androstenedione and testosterone produced and plotted. 5α-reductase activity was measured by combining the amounts of 5α,3α/3β-P (Panel C). The ratios of 17-OHP to total of 17-OHP, androstenedione, and testosterone are shown plotted (Panel D). Significant differences between the aggregate ratios are shown; $^{*}p < 0.05$ and $^{**}p < 0.001$. Symbols: (▲) 50 μM 8Br-cAMP; (▼) 50μ M 8Br-cAMP and 35 nM MIS; (■) untreated; (♦) 35 nM MIS. Panel E, TLC analysis with ^{14}C-progesterone incubated with testicular microsomes prepared from rats treated with and without MIS as described in Section 2. The migration of steroid standards is indicated on the right.

as a function of progesterone concentration, which begins at 25 nM and continues to 2 μM in the case of cAMP-treated cells. As expected, the velocity of the Cyp17 hydroxylase reaction with microsomes from cAMP-treated MA-10 cells was more than six-fold higher over that of untreated cells (Fig. 6A). Co-incubation of the cells with cAMP and MIS reduced the velocity of the hydroxylase reaction back to the unstimulated state. Treatment of the cells with MIS alone resulted in very little measurable conversion of progesterone to 17-OHP or androstenedione above background. The lyase step in the conversion of 17-OHP to androstenedione is the second step catalyzed by Cyp17 but it does so with 17-OHP as an intermediate that does not dissociate from the enzyme, indicating that progesterone is the proper substrate for assaying the lyase activity [28,29]. Therefore, we assayed Cyp17 lyase activity with progesterone as the substrate. As with the hydroxylase activity, these data also show that the velocity of the lyase activity is greatly enhanced by addition of cAMP to the cells and is similarly reduced to that of unstimulated cells by co-incubation with MIS (Fig. 6B).

We also measured the effect of cAMP and MIS on the production of 5α-reduced progesterone to determine whether the effect of MIS was specific to Cyp17 activity (Fig. 6C). As expected, unstimulated MA-10 microsomes metabolize progesterone to its 5α-reduced forms with a greater velocity than is seen with microsomes from cAMP-stimulated cells or from cells treated with both cAMP and MIS. However, unlike the reversal of cAMP-stimulated Cyp17 activity observed with added MIS, a marginal effect is seen on 5α-reductase activity.

Because of the number of products formed in the microsomal assays and because the assays were performed to completion, Michaelis–Menton kinetics could not be accurately assessed with the data collected. However, assessing the ratios of the 17-OHP released by Cyp17 to the 17-OHP converted to androstenedione reflects the activities of the enzyme and are shown in Fig. 6D. Untreated MA-10 microsomes produced approximately equal amounts of 17-OHP and androstenedione at the lowest concentration of progesterone (25 nM) but the amount of 17-OHP decreased to 25% of the total at the higher concentrations. In contrast, the amount of 17-OHP was never greater than 20% of the total with the microsomes from cells treated with cAMP and at the higher concentrations of progesterone substrate was approximately 10%. Addition of MIS and cAMP to the cells resulted in microsomes that also produced a higher ratio of 17-OHP, approximately 38%, at the lower concentrations, which was reduced to less than 20% at the higher concentrations of progesterone substrate when compared to addition of cAMP alone. The data from microsomes of MA-10 cells treated with MIS alone were too low to be interpreted (data not shown).

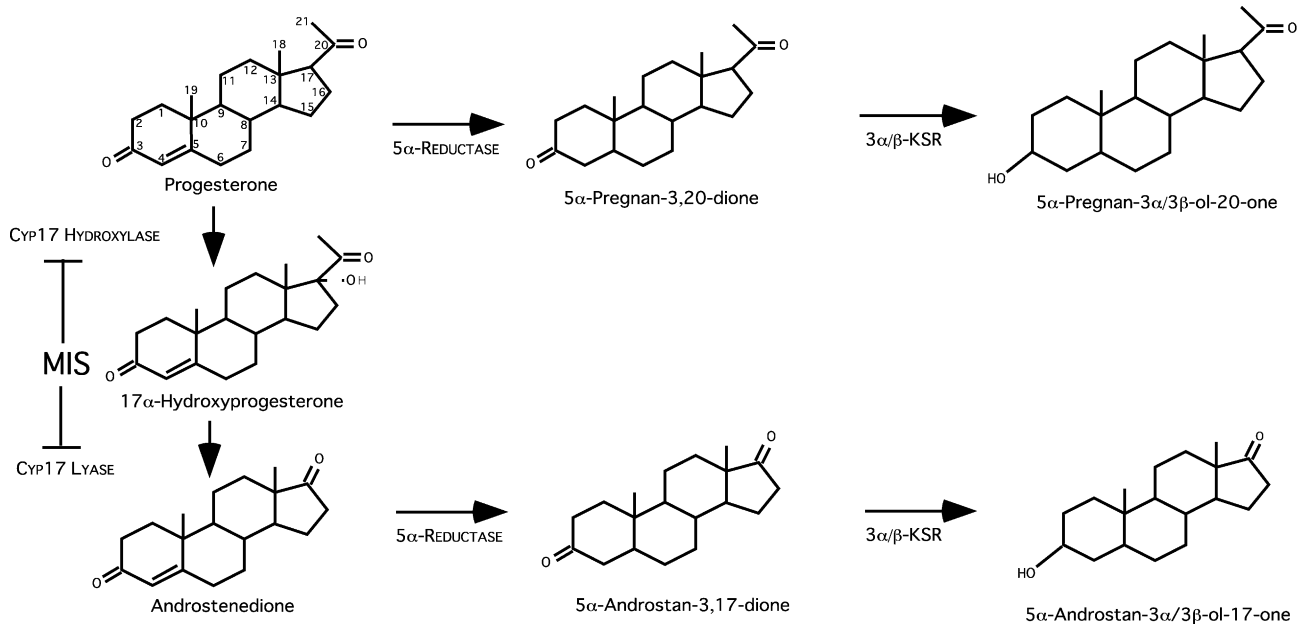

Fig. 7. MA-10 steroidogenic pathways in response to cAMP or MIS. In MA-10 microsomes, progesterone is normally converted to its 5α-reduced metabolites. If the cells are treated with cAMP and Cyp17 expression is induced, progesterone is converted to androstenedione and its 5α-reduced forms. MIS, when added to cAMP-stimulated cells, inhibits Cyp17 expression, mitigating the shift of the microsomes to androstenedione production. Carbons are shown numbered in the progesterone molecule.

A TLC of rat testicular microsomes with progesterone substrate was performed to determine whether MIS affects the Cyp17 lyase activity in vivo (6E). Unlike MA-10 cells, the main metabolites of rat testicular microsomes were androstenedione, DHT, and testosterone. Conversely, the ability of microsomes from MIS-treated rats to convert progesterone was greatly reduced and 17-OHP appears to be the major metabolite. As with MA-10 cells, treatment with MIS inhibited Cyp17 lyase activity, which in the testicular microsomes resulted in greatly reduced production of the downstream products, androstenedione, testosterone, and DHT and relatively more 17-OHP production. These results suggest that MIS inhibits Cyp17 lyase activity in vivo, as well as in MA-10 cells.

4. Discussion

Steroidogenesis in Leydig cells has been well studied (for reviews see [22,31,32]). However, the paracrine control of steroidogenesis in Leydig cells by MIS has only recently been addressed. MIS inhibits androgen synthesis in Leydig cells largely by inhibiting the expression of Cyp17 both in vitro and in vivo [2] probably by interfering with PKA signaling [6] but the exact mechanisms involved are still unknown.

The 5α-reduced forms of progesterone are the major steroid hormones produced by MA-10 cells [21]. By inducing Cyp17 expression, cAMP essentially switches the MA-10 Leydig cell from being a cell producing predominantly 5α-reduced progesterone metabolites to one that is much more efficiently converting progesterone to androstenedione and its metabolites (Fig. 7). This is observed in spite of the unchanged 5α-reductase activity in the microsomes since neither addition of cAMP or MIS appears to affect the expression of 5α-reductase type 1 (Fig. 3). A possible explanation for this observation is that progesterone is a better substrate for Cyp17 than it is for 5α-reductase. The K_m of rat Cyp17 has been reported in the 26–79 nM range [33] while the K_m for 5α-reductase is in the low μM range [34], supporting this hypothesis.

As opposed to the Cyp17, MIS does not appear to affect greatly the 5α-reduced progesterone, which has been described as a salvage pathway for progesterone that may be necessary for its rapid elimination during cervical ripening and parturition [35–37]. In MA-10 microsomes, the 5α-reduced progesterone appears to be rapidly metabolized by 3β-reduction but the physiological significance of this activity is not clear. In the brain, 5α,3α-P is a potent neurosteroid sedative [38] whose binding to the GABA receptor can be blocked by its epimer, 5α,3β-P [39]. The physiological significance of 5α,3β-P production by Leydig cells and their responses to cAMP and MIS are unknown.

Activation of PKA by cAMP and its subsequent downstream signaling cascade induces Cyp17 expression and Cyp17 phosphorylation in Leydig cells; the Cyp17 phosphorylation has been proposed to increase its lyase activity [40,41]. Our results suggest that cAMP and MIS might be altering the kinetics of either the Cyp17 hydroxylase or lyase activities. We are unaware of any published studies comparing the kinetics of Cyp17 in the presence or absence of

cAMP. However, Miller and colleagues have reported that overexpression of human cytochrome P450 oxidoreductase with human Cyp17 in yeast results in a 10-fold higher Vmax and a 50% higher K_m with progesterone substrate [42]. Perhaps activation of the PKA signaling pathway decreases the dissociation rate of 17-OHP from Cyp17 thus favoring its cleavage to androstenedione. In order to study changes in the Michaelis–Menton kinetics of Cyp17 activity with cAMP and/or MIS signaling, similar additional studies would be required.

Differential effects on Cyp17 activities by cAMP and their reversal by MIS may be a mechanism allowing LH-stimulated Leydig cells to continue to produce relatively higher amounts of 17-OHP for use in other steroidogenic pathways after achieving optimal levels of androstenedione, a more committed steroid intermediate. For example, a recent report by Wilson and colleagues has shown that 5α-reduced 17-OHP is an intermediate in one of two pathways used to produce the predominant androgen 5α-androstane-3α,17β-diol in testes of early tammar wallaby pouch young [35]. Although Cyp17 lyase activity was still required later on in the pathway for androgen production in this case, the relatively less modified 17-OHP was a good substrate for use by another enzyme. This is particularly important in human adrenals where 17-OHP is converted to cortisol by the activity of P450c21 and P450c11 hydroxylases. A role for MIS in human adrenals awaits further studies.

MIS inhibits androgen synthesis in Leydig cells largely by inhibiting the expression of Cyp17 both in vitro and in vivo [2] probably by interfering with PKA signaling [6] but the exact mechanisms involved are still unknown. In this report we have presented evidence that suggests that MIS also differentially modulates the Cyp17 hydroxylase and lyase activities. Addition of cAMP to MA-10 cells appears to cause less 17-OHP to be released from the Cyp17 active site, which results in the subsequent increase of Cyp17 lyase activity and androstenedione production (Fig. 3). Addition of MIS to the cells partially reverses the cAMP-mediated inhibition release of 17-OHP from Cyp17 resulting in relatively higher levels of 17-OHP. Based on our results, we speculate that, in addition to the ability of MIS to inhibit cAMP-mediated Cyp17 expression, MIS affects the phosphorylation state of Cyp17 itself so that the lyase activity is less efficient.

Over expression of MIS or perturbation of the MIS signal transduction causes mice to develop severe gonadal abnormalities [16,43] that are inconsistent with simple androgen insufficiency phenotypes, suggesting functions for MIS that are independent of androgen-mediated effects. Based on our results, one could speculate that before puberty, when MIS expression in the testis is high, steroidogenesis in Leydig cells is shunted towards the 5α-reduced progesterone metabolites by the ability of MIS to inhibit Cyp17 expression, adding yet another layer of complexity to the physiological role played by MIS after Müllerian duct regression.

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