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Implications of Progesterone Metabolism in MA-10 Cells for Accurate Measurement of the Rate of Steroidogenesis

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In virtually all studies with MA-10 cells, progesterone RIAs have been used to measure steroid synthesis. To test whether progesterone is a stable end product, we investigated the metabolism of added tritiated progesterone and pregnenolone in MA-10 cells over a period of 3 h. Steroids were then extracted, separated by HPLC, and identified by GC/MS. We found that more than 70% of radiolabeled steroids were converted to at least five different metabolites. A major metabolite (40%) was 5α-pregnan-3α or 3β-ol-20one. Similar studies, using radiolabeled T, demonstrated conversion to dihydrotestosterone and two forms of 5α-androstane-diols. These data indicate the presence of active 5α-reductase and 3α- and/or 3β-hydroxysteroid dehydrogenase activities in MA-10 cells.

Because these results suggest that progesterone is an unstable end product, to gauge the level of active metabolism, we incubated cells in the presence of inhibitors of pregnenolone metabolism and assessed pregnenolone levels by RIA. We discovered that basal levels of steroidogenesis in MA-10 cells were considerably higher than previously estimated. Moreover, dibutyryl cAMP-stimulated steroid production was linear over more than 13 h, in contrast to previous findings that measured progesterone levels. Other consequences of inaccurate assessment of steroidogenic activity in MA-10 cells because of the application of the progesterone assay are discussed. (*Endocrinology* 142: 5236–5242, 2001)

THE MOUSE LEYDIG tumor cell line MA-10 is widely used as a model system for investigations on the regulation of steroidogenesis. The cells can be grown in culture and possess functional LH/human CG (LH/hCG) receptors that mediate a very strong stimulation of progesterone production (1). The cells show all the features of normal Leydig cells, except that the amount of P450c17 mRNA and P450c17 activity is insignificant (2). Consequently, the production of androgens by these Leydig cells is almost undetectable; and instead, large quantities of progesterone are produced (1, 2). Because only small amounts of 20α-dihydroprogesterone are produced and few other metabolites have been identified during short-term incubations, changes in the levels of progesterone, as determined by RIA, have been used as a standard measure for the overall steroidogenic activities of MA-10 cells. This procedure has been used by nearly all investigators over a period of 15 yr. However, it has been reported that accumulated progesterone in medium of stimulated MA-10 cells is decreased to almost basal levels after 24 h of incubation (3). This clearly indicates active metabolism of progesterone. Although the linear increase in progesterone levels in the incubation medium, by stimulated MA-10 cells, during the first hours suggests that progesterone is not metabolized at this time, progesterone levels do not further increase after 6–8 h (1, 4–6). This has been widely interpreted to mean that with long-term stimulation, there is an exhaustion of steroid-synthesizing capabilities or a desensitization to trophic stimulus in MA-10 cells, resulting in a decline in steroidogenesis. However, levels of 20α-dihydroprogesterone increase linearly over at least 12 h (1). Moreover, normal Leydig cells isolated from mouse testes do not show such a tendency for decreased steroid production, not even after 24 h of stimulation (7, 8). The diminished accumulation of progesterone after 6–8 h could also result from an increased influence of progesterone metabolism. Although only a limited variety of progesterone byproducts have been identified, major metabolites may have escaped detection because of limited separation power of the previously used chromatography systems (1, 5). If the level of progesterone metabolism is indeed significantly underestimated, progesterone assays will not give an accurate measure of total steroidogenesis in MA-10 cells. In fact, they could provide misleading data.

We (see Refs. 10 and 11) have previously established conditions to measure total steroidogenesis in immature rat Leydig cells, which are very active in the metabolism of T (9). This was possible by completely inhibiting the metabolism of pregnenolone without affecting its biosynthesis (10, 11). In the presence of these inhibitors, the only steroidogenic product made by these rat Leydig cells is pregnenolone. By measuring this immediate end product of cholesterol side-chain cleavage activity, we were able to measure the true rate of steroidogenesis in rat Leydig cells without complications of steroid metabolism. In a study on LH receptor activation by different hCG preparations and using MA-10 cells, we found that the measured biological activity of the various hCG preparations depended on the type of RIA (for progesterone or for pregnenolone) used for measuring steroid production (12). We suggested that the discrepancies between the results of the two assays could result from progesterone metabolism. For this reason, we have reinvestigated the steroid metabolic

Abbreviations: AMG, Aminoglutethimide; dbcAMP, dibutyryl cAMP; hCG, human CG.

enzyme activities in MA-10 cells. We have also evaluated whether the pregnenolone method, previously used for rat Leydig cells, can be applied to MA-10 cells and also how this method compares with the progesterone assay.

Materials and Methods

RPMI-1640, horse serum, and FCS were from Grand Island Biochemical Co. (Grand Island, NY). BSA was from Sigma (St. Louis, MO). Diethylether, n-hexane, and 2-propanol (all LiChrosolv) were purchased from Merck & Co., Inc. (Darmstadt, Germany). Highly purified hCG was kindly provided by Organon International (Oss, The Netherlands). SU-10603 [7-chloro-3,4-dihydro-2-(3-pyridyl)-1-(^{2}H)-naphthalenone] was a gift from Ciba-Geigy (Basel, Switzerland). Epostane or WIN-32729 (4α,5α-epoxy-17β-hydroxy-4β,17α-dimethyl-3-oxo-androstane-2α-carbonitrile) was a gift from Sterling-Winthrop (New York, NY). Finasteride [17β-(*N*-tert-butylcarbamoyl)-4-aza-5α-androstan-1-en-3-one] was a gift from Merck & Co., Inc. (Rahway, NJ). [7-^{3}H]-Pregnenolone, [1,2,6,7-^{3}H]-progesterone (3.37 TBq/mmol), [1,2,6,7-^{3}H]-T (3.63 TBq/mmol) were purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK).

MA-10 cells (1) were generously donated by Dr. Mario Ascoli (University of Iowa, Iowa City, IA). Cells were grown under standard conditions in RPMI-1640 medium supplemented with 10% horse serum, 100 μg/ml streptomycin sulfate, 100 IU/ml penicillin, and 2 mM L-glutamate, at 37 C, under humidified atmosphere containing 95% air-5% CO_2. The cells were subcultured after trypsin treatment (0.05% wt/vol trypsin), and experimental cultures were plated and cultured for 2 d. After washing twice with RPMI-1640 containing 0.1% BSA, approximately 10^6 cells were used per experiment.

Steroids were measured in the incubation medium and/or in the cells after incubation at 37 C for various periods in RPMI-1640 medium or in Krebs Ringer buffer solutions, containing either 0.1% BSA or 1% FCS. Depending on the type of experiments, epostane (5 μM) was added from stock solution to inhibit 3β-hydroxysteroid dehydrogenase activity. In some experiments, 20 μM SU-10603 was also added to inhibit residual activities of 17α-hydroxylase/desmolase. Steroids in culture medium (300 μl) or sonified cells (see below) were measured with RIAs previously described for pregnenolone (10, 11), 20α-dihydroprogesterone (13), and progesterone (14). The steroid specificities of the three antisera used are given in Table 1. For measurement of cellular steroid levels, cells were washed with RPMI-1640 medium and were trypsin treated (0.05% wt/vol). After sonification, 300 μl of the resulting homogenate was used for measurement of steroid levels. For investigation of progesterone, pregnenolone, or T metabolism, 0.03–30 nmol ^{3}H-labeled steroids were added to the culture medium. After incubation for 3 h, culture medium was extracted three times with ethyl acetate, and the metabolites were separated by HPLC with online radiodetection as described previously (15). In short, metabolized steroids were separated on a Hibar LiChrosorb Diol-column (length, 250 mm; diameter, 5 μm; Merck & Co., Inc., Rahway, NJ), equipped with a guard column (Resolve Silica, Waters Corp., Milford, MA). The HPLC-system included a Waters 610 Fluid Unit, a Waters 600E System Controller and a Waters U6K injector. The isocratic flow of the mobile phase (hexane/propanol 96:4, vol/vol) was 1.5 ml/min. Radioactivity was monitored with a FloOne β Radiomatic A500 radiochromatography detector (Packard-Canberra Benelux, Tilburg, The Netherlands) with a 500-μl cell and a liquid scintillation flow of 1.5 ml/min (Aqua-Luma, Lumac-LSC, Olen, Belgium).

For identification of an unknown major metabolite, various batches of MA-10 cells were incubated for 3 h, with 30 nmol pregnenolone per 10^6 MA-10 cells, until approximately 10 μg pregnenolone-like steroids could be measured in the combined ethylacetate extracts. During the HPLC separation procedure, the major metabolite was collected. After trimethylsilyl-enol-trimethylsilyl derivatization, steroid fractions were directly used for gas chromatographic/mass spectrometric (GC/MS) analysis. GC/MS analysis was performed on a 5790 Gas Chromatograph (Hewlett-Packard Co., Palo Alto, CA) coupled to a 5970 Mass Selective Detector (MSD, Hewlett-Packard Co.). Structural information about the major metabolite was obtained by comparing the mass spectrum of the metabolite with those of authentic reference steroids. Further details about this procedure can be found in Ref. 16.

Data are expressed as means ± SD and analyzed for statistical significance using the *t* test. Values of *P* less than 0.01 were considered significant.

Results

Steroidogenesis, under basal conditions and during stimulation with hCG, was measured by incubating MA-10 cells, without or with epostane and SU-10603, to determine to what extent pregnenolone metabolism is inhibited. The secreted steroids in the culture media were measured with three different RIAs, and the results are presented in Table 2. Results from the progesterone assay confirm what has been observed previously for MA-10 cells: control cells secrete very small amounts of progesterone, and the production is increased approximately 200-fold after hCG stimulation. When MA-10 cells were incubated in the presence of epostane and SU-10603, which completely inhibit 3β-hydroxysteroid dehydrogenase and 17α-hydroxylase/desmolase activity in rat Leydig cells, progesterone synthesis was reduced by nearly 85%. The data further confirm that MA-10 cells also, in addition to progesterone, produce 20α-dihydroprogesterone. The generation of this steroid was similarly stimulated by hCG and affected by the presence of these inhibitors of pregnenolone metabolism. When MA-10 Leydig cells were incubated with these inhibitors and the RIA for pregnenolone was applied, higher amounts of steroids were measured than with the progesterone assay. Surprisingly, when epostane and SU-10603 were omitted, there was only a marginal effect on the outcome of the pregnenolone assay. This is particularly remarkable because the inhibitors were active, as could be concluded from the concomitant attenuation of progesterone production. This apparent discrepancy can be understood if pregnenolone-like metabolites are formed that interact with the antipregnenolone antibody (Table 1).

TABLE 1. Steroid specificity of three RIAs

Cross-reacting steroid	Antibody against steroid-BSA conjugate (BSA conjugation position on steroid)		
	Pregenolone (3)	Progesterone (11)	20α-OH Progesterone (11)
5-pregnen-3β-ol-20one	100	<1	ND
5-pregnen-3β, 17-diol-20one	8	ND	<1
4-pregnen-3,20dione	20	100	1
4-pregnen-20α-ol-3one	<5	<1	100
4-pregnen-3β,17-diol-20one	8	2	<1
4-pregnen-3β,11β-diol-20one	<1	30	<1
4-pregnen-11β,17,21-triol-3,20dione	29	<1	<1
5α-pregnan-3,20dione	20	11	1
5α-pregnan-3α/β-ol-20one	50	ND	ND
4-androsten-17β-ol-3one	<1	<1	<1

TABLE 2. Steroids (pmol) measured in incubation medium after incubation of 4×10^5 MA-10 cells for 3 h without or with 0.1 nM hCG

Condition RIA	Without inhibitors			With inhibitors EP and SU		
	Progesterone	20α-Progesterone	Pregnenolone	Progesterone	20α-Progesterone	Pregnenolone
Control (a)	2.6 ± 0.1	0.8 ± 0.1	7.9 ± 0.2	2.7 ± 0.1	0.6 ± 0.1	15.6 ± 0.4
hCG (b)	510 ± 43[a]	54 ± 2[a]	620 ± 75[a]	80 ± 3[a]	6.4 ± 0.3[a]	680 ± 52[a]
Approx. ratio b/a	200	70	80	30	10	40

Results from three different RIAs are shown. Steroids were measured in plain incubation medium and in medium containing a mixture of 5 μM epostane (EP) and 20 μM SU-10603 (SU), which inhibit, in rat Leydig cells, 3β-hydroxysteroid dehydrogenase activity and 17α-hydroxylase/desmolase activity, respectively. The steroid specificities of the three different antibodies are shown in Table 1. Mean results ± SD of four to eight observations are shown.

[a] Significantly different from controls ($P < 0.01$).

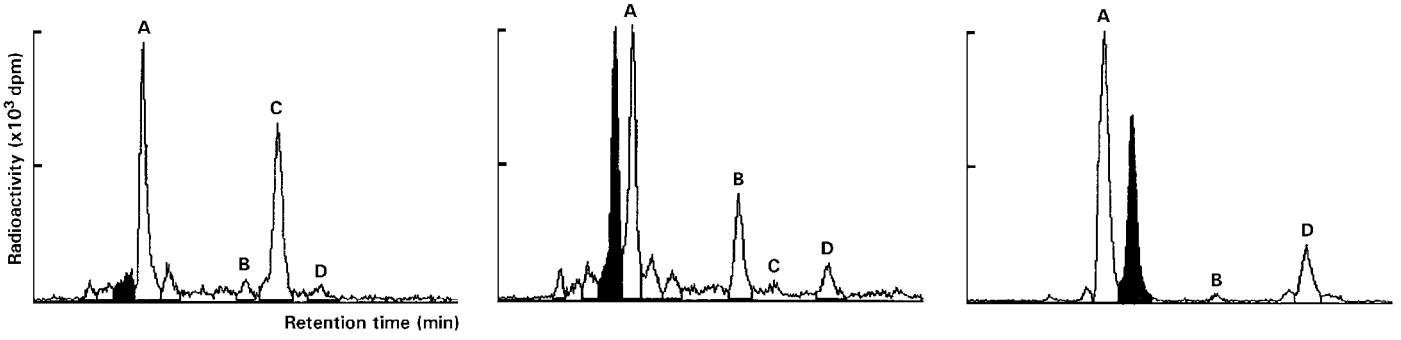

FIG. 1. Metabolism of 0.03 nmol ^{3}H-labeled progesterone (*left*), 30 nmol ^{3}H-labeled progesterone (*middle*), or 0.03 nmol ^{3}H-labeled pregnenolone by 10^6 MA-10 cells in 3 h. Metabolism of pregnenolone occurred in the presence of 5 μm epostane (EP) and 20 μm SU-10603 (SU). Steroids were extracted from the culture media and separated by HPLC. The *black peak* indicates the substrate that remained after the incubation. The distribution of radioactivity over the four mean peaks A, B, C, and D is given in Table 3. In this table, results from other studies on pregnenolone metabolism are also shown.

TABLE 3. Distribution of radioactivity over metabolites, as indicated in Fig. 1[a]

Substrate	nmol	(inhibitors)	% Prog	% Polon	% A	% B	% C	% D	% Others
Prog	0.03		7		37	4	35	4	13
Prog	30		31		31	14	1	6	17
Polon	0.03	(SU+EP)	2	30	56	1		10	1
Polon	0.03	(SU+EP+Fi)	7	41	38	6		3	5
Polon	0.03		2	6	39	2		50	1
Polon	30		18	10	40	18		11	3

[a] Metabolism was studied with different amounts of substrate and inhibitors. For further information, see Fig. 1 legend. Fi, 5 μM finasteride; Prog, progesterone; Polon, pregnenolone.

The metabolism of radioactively labeled pregnenolone and progesterone was therefore investigated under conditions that are frequently employed in studies with MA-10 cells. When cells were incubated for 3 h with low amounts of progesterone that are similar to basal levels (0.03 nmol), more than 95% of the steroid was metabolized (Fig. 1). When 1000-fold more progesterone was added, a level similar to that present when cells are maximally stimulated, approximately 70%, was metabolized during 3 h of incubation. These data show that added progesterone is extensively metabolized by MA-10 cells and a major metabolite that is formed is compound A. Moreover, the extent of this metabolism and the pattern of the metabolites depends on the concentration of progesterone or pregnenolone used (Fig. 1 and Table 3). When cells are incubated with pregnenolone, a major part of this precursor is also converted to compound A, and only a minor fraction to progesterone. In the presence of epostane and SU-10603, there was a higher yield of pregnenolone, but the major steroid present was still compound A, not pregnenolone. Higher concentrations of these drugs failed to further inhibit pregnenolone metabolism (data not shown).

To discover the identity of compound A, this steroid product was collected by an HPLC separation procedure. Mass-spectrometry analysis determined this compound to be either 5α-pregnan-3β-ol-20one or 5α-pregnan-3α-ol-20one, indicating that 3α and/or 3β-hydroxysteroid dehydrogenase and 5α-steroid reductase enzyme activities are very active in MA-10 cells. These enzyme activities, together with the known Δ^5-Δ^4 isomerase and 20α-hydroxysteroid dehydrogenase activities, can give rise to many different products. We have not attempted to characterize the remaining metabolites; but, to measure the 3α and/or 3β-hydroxysteroid dehydrogenase and 5α-steroid reductase enzyme activities without interference of Δ^5-Δ^4 isomerase and 20α-hydroxysteroid dehydrogenase activities, steroid metabolism was also investigated using T as a substrate. The results in Fig. 2 and Table 4 show that T was metabolized to dihydrotestosterone, 5α-androstane-3α,17β-diol, and probably 5α-andro-

stane-3β,17β-diol. From these results it can again be inferred that both 3α/β-hydroxysteroid dehydrogenase and 5α-steroid reductase enzyme activities are active in MA-10 cells. Similar to when progesterone or pregnenolone was used as a substrate, the percent conversion of T decreased when higher concentrations of substrate were used.

Because 5α-steroid reductase is a major activity and 5α-reduced steroids could be preferential substrates for 3α- or 3β-hydroxysteroid dehydrogenases, we also attempted to inhibit conversion of radiolabeled pregnenolone through this metabolic pathway, by inclusion of finasteride in the mixture of inhibitors. At a concentration of 5 μM, 5α-steroid reductase activity in MA-10 cells was indeed inhibited (Table 3). Although, under these conditions, more pregnenolone was recovered and less metabolites were formed, the inhibition of pregnenolone conversion was still incomplete. We have not used this compound as an additional inhibitor for measuring endogenous steroid production, because 3 μM finasteride can reduce cytochrome P450scc enzyme activity by more than 50% in MA-10 cells (17).

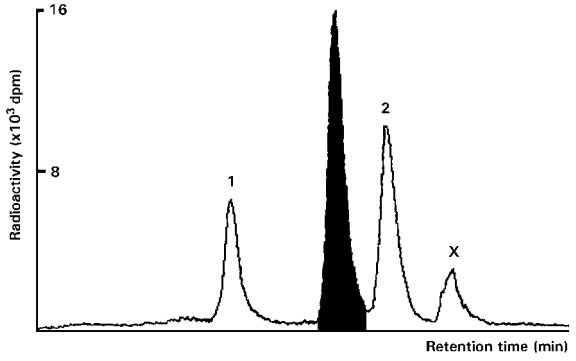

FIG. 2. Metabolism of 0.3 nmol ^{3}H-labeled T by 10^6 MA-10 cells in 3 h. Two peaks could be identified with standards: 1 is dihydrotestosterone (DHT) and 2 is 5α-androstane-3α,17β-diol (3α Adiol). Peak X was not identified but is probably 5α-androstane-3β,17β-diol. The distribution of radioactivity over the four fractions is given in Table 4.

TABLE 4. Distribution of radioactivity over four fractions, as indicated in Fig. 2

Substrate	(nmol)	% Test	% DHT	% Adiol	% X
Test	(0.03)	37	17	38	7
Test	(0.3)	45	17	30	8
Test	(30)	75	7	11	7

Because the 5α-pregnan-3α/β-ol-20one isomers, progesterone (4-pregnen-3,20-dione), and 5α-pregnan-3,20-dione have 50%, 20%, and 20% cross-reactions, respectively, with the antipregnenolone antibody, the levels of these steroids are also partly measured with the pregnenolone RIA. Using this nonspecific assay, the total endogenous production of progesterone, pregnenolone, 5α-pregnan-3α/β-ol-20one and possibly other pregnenolone-like steroids seemed to be linear up to 13 h (Table 5). A substantial fraction of this steroid mixture was cellular-bound; and therefore, this fraction cannot be ignored when assessing total steroid synthesis. The results also showed that, in the presence of the cholesterol side-chain cleavage enzyme inhibitor aminoglutethimide (AMG) (18), endogenous steroid production is inhibited by more than 99%. Thus, the nonspecific pregnenolone assay does not measure nonsteroidal compounds in the culture medium. When MA-10 cells were preincubated in the presence of AMG and dbcAMP for 13 h, upon removal of AMG, the rate of steroidogenesis was 2-fold higher than in MA-10 cells treated for only 2 or 4 h with dbcAMP alone (Table 5, ratio d/a or d/b). This indicates that the rate of steroid production after long-term stimulation of MA-10 cells does not decrease but shows a clear tendency to increase.

Discussion

The present data show that added progesterone can be metabolized by MA-10 cells to such an extent that it can be present as a minor steroid, when compared with its metabolites. This metabolism was measured after the addition of labeled progesterone, as a large bolus, to the culture medium. It is possible that endogenous steroid precursors that are continuously produced in low amounts, close to steroid-converting enzymes in the endoplasmic reticulum, may be preferentially metabolized, because these enzymes show a preference for particular substrates (19, 20).

If this occurs in our experiments, the pattern of steroid metabolites from added labeled pregnenolone or progester-

TABLE 5. Endogenous steroids (nmol) produced by 0.5×10^6 MA-10 cells after different incubation periods

Incubation period (h)	dbcAMP (1 mM)	Epostane (5 μM)		Medium	Cells	Total	Ratio
13	−	+		0.03 0.07	0.04 0.04	0.07 0.1	
2	+	+	(a)	1.1 0.9	0.9 1.1	1.9 2.0	
4	+	+	(b)	2.9 2.3	2.0 2.0	5.0 4.3	b/a 2
13	+	+	(c)	7.4 6.7	6.5 7.0	14 14	c/a 7
13 with 500 μM AMG	+	−		0.03 0.03	0.08 0.06	1.1 1.0	
2 after 13 h preincubation with 500 μM AMG and subsequent removal of the AMG	+	+	(d)	1.9 1.9	1.4 2.4	3.4 4.3	d/a 2
13	+	−	(e)	3.3 3.1	0.3 0.4	3.6 3.5	

one may be slightly different from the endogenously produced steroids. Still, the observed metabolism of added progesterone, pregnenolone, or T clearly shows that there are active steroid metabolizing enzymes present in MA-10 cells. Irrespective of the exact profile of the metabolites, active metabolism of progesterone will lead to an underestimation of the true steroidogenic activities of the MA-10 cells. A similar problem was previously encountered when the steroidogenic activity of immature rat Leydig cells (9) or rat H540 tumor cells (21) was measured with an RIA for T. Unlike mature Leydig cells, immature rat Leydig cells and rat H540 tumor cells are very active in metabolizing T; and hence, the measurement of T as an end product of steroid synthesis grossly underestimates the steroidogenic activity of these cells. Accurate analysis of steroidogenesis in these rat Leydig cells, however, is possible because metabolism of pregnenolone can be abolished after inhibitors are added and pregnenolone accumulation is assessed by RIA (10, 11). These inhibitors also reduced pregnenolone conversion in MA-10 cells, but the inhibition was incomplete. However, because the major metabolites, 5α-pregnan-3α/β-ol-20one and progesterone, also partly cross-react with the antipregnenolone antibody, this nonspecific RIA gives a better, though not perfect, impression of the total steroidogenic activity than can be derived from specific progesterone measurements. Similar findings were reported by Purvis *et al.* (9) in 1978, when they were able to show hCG responsiveness of immature Leydig cells with a nonspecific 17β-hydroxyandrogen assay but not with a specific T assay. Unfortunately, our attempts to improve the reliability of the pregnenolone assay for MA-10 cells by adding finasteride to further inhibit pregnenolone metabolism were unsuccessful. Although the pregnenolone assay is imperfect, it gives a much better impression about steroid production than can be derived from progesterone assays. The discrepancies between the two assays seemed most pronounced when low amounts of steroid were produced, as from unstimulated MA-10 Leydig cells, or when high amounts of steroids were produced, when cells are chronically stimulated. Some examples and consequences of inaccurate assessment of steroidogenic activities in MA-10 cells in the literature are discussed below.

Our observations with the pregnenolone assay of unstimulated MA-10 cells show that these cells produce more steroids than previously thought. The reported very low progesterone production by unstimulated MA-10 cells (1, 4, 5) thus seems to be more a reflection of a high metabolic rate than of a very low rate of steroidogenesis. If small amounts of progesterone are almost completely metabolized, this could also explain why progesterone synthesis in MA-10 cells responds relatively slowly to hCG stimulation, when compared with T production in normal mouse (22) or rat (23) Leydig cells.

Because unstimulated MA-10 cells are more active in steroidogenesis than heretofore recognized, this also affects the observed stimulation factor of steroid synthesis induced by the addition of trophic hormones. The stimulation factor in MA-10 cells, using the pregnenolone RIA, was approximately 30. This is very close to the data published for rat Leydig cells but much lower than the more-than-200-fold or higher acute stimulation factors derived from progesterone measurements in MA-10 cells (4, 6, 24). Thus, progesterone assays may give a false impression of the kinetics of steroid production and its responsiveness to hormonal stimulation.

Our observations with the pregnenolone assay showed that stimulated MA-10 cells continue to produce steroids over a period of at least 13 h. This corroborates an earlier observation that the production of 20α-dihydroprogesterone, but not progesterone, is constant for at least 12 h (1). It also seems, from our data, that the rate of steroid production does not decrease over this time but may instead increase, given that steroidogenesis after 13 h is higher than the rate of steroid production during the first 4 h. This is in line with the reported increase in cholesterol side-chain cleavage enzyme activity after 24 h incubation with dbcAMP (24).

Thus, the plateau of progesterone levels in culture medium after incubation of MA-10 cells for 6–8 h (1, 4) most likely results from a high rate of progesterone metabolism and does not originate from a decline in steroid synthesis. This influence of metabolism is often ignored, and changes in progesterone levels are often interpreted to be a direct consequence of changes in steroidogenesis. For instance, changes in progesterone have been presented, in a relative fashion, as a percentage of maximal expression, with the maximal level of progesterone taken as 100%, and these relative steroid values have been compared with StAR mRNA levels (4). Because progesterone and StAR mRNA levels develop in a similar fashion and both reach a plateau after approximately 6 h, it was concluded that the rate of StAR mRNA synthesis parallels that of steroid production. However, when progesterone metabolism is taken into account, the plateau probably more accurately reflects a balance between metabolism and production, not a slowdown in steroid synthesis. In addition, using the pregnenolone assay, the present data show that the true steroidogenic activity and StAR mRNA levels do not correlate after 6 h. Presumably, the early accumulation and later decline in StAR mRNA levels should yield changes in the levels of StAR protein and, thus, changes in the rate of steroid production. However, our data from the pregnenolone assay, as well as the data from the progesterone assays up to 6 h, revealed a linear increase in steroid levels and thus a constant rate of steroidogenesis. Therefore, the significance of changes in StAR mRNA levels for steroidogenic activity remains unclear at this time.

Discrepancies between the apparent and the real steroidogenic activity are very clear after prolonged incubation periods, because progesterone levels in media from stimulated cells not only reach a plateau but also diminish after 6 h of stimulation (3). Because of this metabolism of progesterone, the apparent level of steroidogenesis in MA-10 cells induced by LH was found to be 10-fold less when an incubation period of 24 h was used instead of 4 h (3).

Because of the observed discrepancies between real and apparent steroidogenic activities, not only during prolonged incubation periods of stimulated cells but also during short-term incubations of unstimulated cells, it remains to be determined whether reliable information can be obtained concerning the fine regulation of steroidogenesis from progesterone measurements. By using normal mouse Leydig cells and measuring T levels, important information on the

regulation of steroid production was obtained from careful kinetic studies over 4- to 5-h periods (22). It was shown that very low concentrations of hCG that do not give detectable changes in cAMP and PKA activation can almost maximally stimulate T production, but the increase in this steroidogenic activity is only detectable after a delay of 3–4 h (22). The authors concluded from their kinetic experiments that submaximal stimulation of steroidogenesis is more the result of a delayed onset than of a reduced rate. The slow increase in the rate of T production, against a background of basal steroid production, could only be measured because T was not metabolized. Because progesterone is not a stable end product, this important conclusion about dose-dependent regulation of steroid production cannot be validated for MA-10 cells. It seems necessary to do this because it has been suggested that considerably higher cAMP concentrations are required for acute stimulation of steroidogenesis than for synthesis of P450scc in MA-10 cells (24). This conclusion was made after incubating MA-10 cells, for 24 h, with 10 μM dbcAMP and measuring progesterone accumulation and P450scc induction.

From the available evidence, it is now clear that, during prolonged incubations of stimulated MA-10 cells, many (mainly pregnane-derived) steroids accumulate, because of the metabolism of progesterone. In a recent publication on the inhibitory effect of Müllerian inhibiting substance on the induction of steroidogenic enzymes in MA-10 cells, it was shown, for the first time, that the *cyp17* gene can be activated strongly in MA-10 cells after 18 h of stimulation with 50 μM dbcAMP (25). This is in contrast to previous findings that *cyp17* gene and 17α-hydroxylase/desmolase activity were absent in MA-10 cells and could not be induced after exposure to dbcAMP (2). Teixeira *et al*. (25) also showed that this induction was completely quenched in the presence of müllerian inhibiting substance. However, the reported shifts in this gene activity, after 18 h of incubation, were not reflected in changes in progesterone levels. Because the levels of progesterone at 24 h and 48 h were low and not different, there was no indication of active steroid production. Only a small increase in the very low levels of T was observed after 48 h. From our study with MA-10 cells and also from incubation studies in the past with immature rat Leydig cells (20) and H540 rat tumor Leydig cells (21), it is evident that there are many possibilities for steroid metabolism. Chronic exposure to dbcAMP could not only further increase the activity of 17α-hydroxylase/desmolase in MA-10 cells, but that of other enzymes as well. Under these conditions, it seems almost impossible to relate changes in T levels to changes in *cyp17* gene activity.

Since their introduction, MA-10 cells have proven very useful for studies on hormonal regulation of steroidogenesis. However, as this paper shows, accurate measurement of steroidogenesis because of progesterone metabolism is a limiting factor. The described pregnenolone assay gives a better impression about the real steroidogenic activity than the progesterone assay; but, for a truly accurate assessment of steroid synthesis, further improvements in methodology are necessary. The observed underestimation of progesterone metabolism may be attributable to the limited capacity of TLC used in the past to separate steroids. Only 20α-dihydroprogesterone was identified as a major metabolite (1, 5). With HPLC, more metabolites could be identified; but even with HPLC, the major metabolite 5α-pregnane-3α/β-ol-20-one could not be completely separated from progesterone. It is therefore possible that this metabolite was also formed in the MA-10 cells used in the previous studies but could not be detected with the TLC system. Also, it remains to be investigated whether sublines of MA-10 cells with different steroid (progesterone) metabolizing capacities now exist. If indeed, sublines of MA-10 cells exist, with different activities of steroid-metabolizing enzymes, this will further complicate quantitative assessment of steroidogenesis via progesterone measurements.

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