Calcium-dependent, cyclic nucleotide-independent steroidogenesis in the bovine placenta

(progesterone/luteinizing hormone/calcium ionophore/3-isobutyl-1-methylxanthine)

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ABSTRACT

Dispersed bovine placental cells (fetal cotyledon and maternal caruncle) were shown to synthesize progesterone. To determine if their steroidogenic activity could be modulated by a cyclic nucleotide-mediated process, we added luteinizing hormone, 8-bromoadenosine 3',5'-monophosphate, 8-bromoguanosine 3',5'-monophosphate, adenosine, or choline toxin to dispersed cells from placentomes of 100–283 days gestational age and examined progesterone synthesis during 3- to 16-hr incubation periods. Net progesterone production, defined as the amount of progesterone released in excess of the zero-time cellular progesterone content, was determined by using a specific RIA. None of these agents significantly affected progesterone synthesis. In contrast, the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (MIX; 0.2–0.5 mM) caused a marked increase in progesterone formation. In time course studies it was found that MIX produced a 5-fold increase in progesterone production in 16 hr, with steroid production increasing linearly during this time. MIX also increased the conversion of exogenous pregnenolone to progesterone by placental cells. In view of the failure of cyclic nucleotide analogues and activators of adenylate cyclase to stimulate steroidogenesis, it was necessary to consider other modes of action of MIX. Since MIX is known to affect intracellular calcium translocation, we examined the effects of the calcium ionophore A23187 on progesterone formation. This drug enhanced progesterone formation and augmented the stimulatory effects of MIX. The stimulatory action of A23187 was not affected by cyclic nucleotide analogues. Our data suggest that progesterone synthesis in the bovine placenta is calcium dependent and cyclic nucleotide independent.

The bovine placenta has been generally disregarded as a site of progesterone synthesis (1–4). However, conversion of [3H]pregnenolone to radioactive progesterone by bovine placental preparations has been demonstrated (5), and our previous studies indicated that enzyme-dispersed bovine placentomes are capable of producing progesterone in culture (6). The present study was conducted to further define the endocrine activity of the bovine placenta with respect to progesterone production and to elucidate mechanisms by which placental steroidogenesis is controlled. The data presented suggest that calcium is the key mediator of placental steroid hormone synthesis, in contrast to other steroidogenic glands in which cyclic nucleotides modulate hormone formation.

MATERIALS AND METHODS

Animals and Tissues. Placental tissues were collected from monoparous Holstein Friesian cows at known gestational ages (n = 13) and from multiparous Holstein Friesian cows at an abattoir (n = 24). Gestational age in the latter animals was estimated by the crown-rump length of the fetus. Maternal caruncles and fetal cotyledons were separated by blunt dissection.

Culture of Dispersed Placental Cells. Maternal caruncles or fetal cotyledons collected at 90–283 days of gestation were dispersed by collagenase by using a modification of the method of Simmons et al. (7). Dispersed cells (2.5 × 10⁶ cells per vessel) were cultured in plastic 25-cm² flasks (Falcon) in 5 ml of medium 199 (GIBCO) supplemented with 5% calf serum. The cells were maintained at 37°C in an atmosphere of 5% CO₂/95% air. Culture media were changed every 4 days. In each experiment, aliquots of cells were taken on the day of plating to determine cellular progesterone content.

Short-Term Incubations of Dispersed Placental Cells. Incubations of 1–1.5 × 10⁶ cells from dispersed fetal cotyledons and maternal caruncles in 1.2 ml of medium 199 were carried out for a period of 3–16 hr at 37°C in an atmosphere of 5% CO₂/95% air. Progesterone levels were determined in the cells at zero time and again in the incubation medium after the indicated times of incubation. The progesterone levels at zero time ranged from 0.2 to 0.5 ng per 10⁶ cells. Each treatment group consisted of five replicates.

Assay of Progesterone. Progesterone secreted into the incubation medium was quantified in diethyl ether extracts by RIA, as described (8). The interand intraassay coefficients of variation of the progesterone assay were 11% and 9%, respectively. In all cases, net progesterone secretion into the medium was determined by subtracting progesterone levels in the cells and incubation medium at zero time from levels after the indicated incubation periods. The antibody displayed negligible (<1%) cross-reactivity with pregnenolone. Cell viability was determined by trypsin blue exclusion (9).

Statistical Analysis. Analysis of variance, followed by either Dunnett’s test, Student’s t test or, when appropriate, a paired t test, was used for statistical analyses.

Materials. Bovine luteinizing hormone (LH) (NIH LH-B9, National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases) was generously provided by the National Pituitary Agency. TMB-8 [8-(N,N-dimethylamino)octyl-3,4,5-trimethoxybenzoate] was obtained from Calbiochem; other chemicals and reagents were purchased from Sigma.

RESULTS

To define the pattern of progesterone synthesis by both fetal cotyledons and maternal caruncles, placental cells derived from cows at 182–283 days of gestation were cultured in medium 199 supplemented with 5% calf serum. Progesterone synthesis by both fetal and maternal cells was greatest during the first 4 days of culture and then declined (Fig. 1). The in vitro production of progesterone by maternal caruncles was

Abbreviations: LH, luteinizing hormone; MIX, 3-isobutyl-1-methylxanthine; 8-Br-cAMP, 8-bromoadenosine 3’,5’-monophosphate; 8-Br-cGMP, 8-bromoguanosine 3’,5’-monophosphate; TMB-8, [8- (N,N-dimethylamino)octyl-3,4,5-trimethoxybenzoate].

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considerably less, on a per cell basis, than by cotyledonary cells. This pattern of steroid production was observed in 10 experiments using tissues from cows at 182-283 days of gestation.

In view of this indication that placental cells produce progesterone, we decided to determine if the steroidogenic activity of these cells could be modulated by tropic agents.

We examined the effects of additions of LH (100 ng/ml), a hormone that enhances ovarian and testicular steroidogenesis, 8-bromoadenosine 3',5'-monophosphate (8-Br-cAMP; 0.5 mM), and 3-isobutyl-1-methylxanthine (MIX; 0.5 mM) on progesterone synthesis by fetal cotyledon and maternal caruncle cells. As indicated in Fig. 2, cells were exposed to these tropic agents for 3 hr, washed, retreated, and incubated for another 12 hr. This protocol was used to assess both acute and longer-term effects of the factors. Progesterone synthesis in fetal cotyledon cells was not elevated by LH or 8-Br-cAMP. However, addition of MIX during the first and the second incubations resulted in progesterone accumulations 2- to 3-fold higher than was found in control cells at any given time. Addition of LH or 8-Br-cAMP with MIX produced no greater progesterone synthesis than MIX alone (Fig. 3). A similar response was observed when maternal caruncle cells harvested at various stages of gestation were incubated with MIX (Fig. 4).

The time course of progesterone production by dispersed maternal caruncle cells in the presence of MIX was determined (Fig. 5). A concentration of 0.5 mM MIX, which caused maximal stimulation, produced a 5-fold enhancement of progesterone secretion by the cells, with the steroid production being linear during the first 16 hr of incubation. Basal progesterone secretion progressed at a low rate during this period.

Since LH and 8-Br-cAMP did not stimulate placental progesterone synthesis but the phosphodiesterase inhibitor MIX did, we were interested in further exploring the potential role of cyclic nucleotides in the control of steroidogenesis in this tissue. The effects of an analogue of cGMP [8-bromoguanosine 3',5'-monophosphate (8-Br-cGMP)] and factors...
known to stimulate adenylate cyclase (cholera toxin and isoproterenol) were tested (Table 1). None of these treatments significantly increased progesterone synthesis.

![Figure 4](image1.png)  
**Fig. 4.** Effect of MIX on progesterone biosynthesis by incubated dispersed maternal caruncle cells. Values are means ± SEM for net synthesis of five samples from 1 animal at each stage of gestation.

Table 1. Effects of LH, 8-Br-cAMP, 8-Br-cGMP, cholera toxin, and isoproterenol on progesterone synthesis by dispersed maternal caruncle cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Net progesterone secretion, ng per 1.5 × 10^6 cells per 10 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.5 ± 0.19</td>
</tr>
<tr>
<td>LH, 100 ng/ml</td>
<td>7.8 ± 0.20</td>
</tr>
<tr>
<td>8-Br-cGMP, 2 mM</td>
<td>8.1 ± 0.15</td>
</tr>
<tr>
<td>8-Br-cAMP, 2 mM</td>
<td>8.5 ± 0.22</td>
</tr>
<tr>
<td>Cholera toxin, 10 ng/ml</td>
<td>7.78 ± 0.20</td>
</tr>
<tr>
<td>Isoproterenol, 10 μM</td>
<td>7.57 ± 0.34</td>
</tr>
<tr>
<td>+ LH, 100 ng/ml</td>
<td>7.84 ± 0.22</td>
</tr>
<tr>
<td>+ 8-Br-cAMP, 2 mM</td>
<td>8.61 ± 0.30</td>
</tr>
</tbody>
</table>

Cells were incubated for 10 hr in medium 199 (control) in the presence of the indicated additions and net progesterone synthesis was measured. Data are means ± SEM of five replicates in each treatment group from a representative experiment. Each treatment was examined in 3–12 additional experiments with similar results.

We next examined the possibility that the stimulatory effect of MIX is related to translocation of extracellular calcium by studying the calcium ionophore A23187 (10–13) on progesterone production by the placental cells. As can be seen in Table 2, A23187 and MIX each produced 2-fold increases in progesterone production. Furthermore, combination of A23187 and MIX resulted in an additive effect. The effect of A23187 was not potentiated by either 8-Br-cAMP or 8-Br-cGMP. Moreover, basal progesterone synthesis was reduced when TMB-8, a Ca^2+ antagonist that blocks efflux of intracellular calcium without affecting calcium influx (14), was added to the incubation medium. Incubation of the placental cells in a Ca^2+-free medium (Eagle's) also resulted in a decrease in basal progesterone synthesis to 1/5th to 1/10th. Ca^2+ concentrations of 0.0, 0.1, 1.25, and 4 mM resulted in progesterone synthesis (mean ± SEM) of <0.05, 0.1 ± 0.01, 0.27 ± 0.04, and 2.3 ± 0.2 ng per 10^6 cells, respectively (n = 3 animals). Since MIX also acts to block the receptors for adenosine, we studied the ability of adenosine to alter the stimulatory effect of MIX. However, the stimulatory effect of MIX was not altered by the addition of adenosine (Table 2).

To determine if the stimulatory effects of MIX and A23187 on progesterone synthesis are on the conversion of pregnenolone to progesterone, caruncle cells were incubated with exogenous pregnenolone, at a concentration of 100 ng per tube (found to maximally increase progesterone formation), in the presence of MIX and A23187. As can be seen from Fig. 6, pregnenolone, MIX, and A23187 each caused almost 2-fold increases (P < 0.01) in progesterone production. An

![Figure 5](image2.png)  
**Fig. 5.** Time course of basal and MIX-stimulated progesterone synthesis by dispersed bovine maternal caruncle cells. Each point is the mean ± SEM of five replicate samples. These data are representative of 3 experiments.

Table 2. Effects of tropic hormones on the stimulatory effects of A23187 and MIX on progesterone production by bovine caruncle cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Net progesterone secretion, ng per 1.5 × 10^6 cells per 10 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>11.5 ± 0.12</td>
</tr>
<tr>
<td>MIX, 0.5 mM</td>
<td>19.4 ± 0.76</td>
</tr>
<tr>
<td>+ Adenosine, 2 mM</td>
<td>20.9 ± 1.12</td>
</tr>
<tr>
<td>A23187, 3 μM</td>
<td>21.7 ± 0.73</td>
</tr>
<tr>
<td>+ MIX, 0.5 mM</td>
<td>33.3 ± 1.13</td>
</tr>
<tr>
<td>+ 8-Br-cAMP, 3 mM</td>
<td>23.7 ± 0.54</td>
</tr>
<tr>
<td>+ 8-Br-cGMP, 3 mM</td>
<td>22.8 ± 1.19</td>
</tr>
<tr>
<td>TMB-8, 10 μM</td>
<td>7.5 ± 0.2</td>
</tr>
</tbody>
</table>

Cells were incubated for 10 hr with medium 199 (control) in the presence of the various additions and net progesterone synthesis was measured. Data are means ± SEM of five replicates in each treatment group from a representative experiment. The experiment was repeated four times with similar results. Means with different superscripts are significantly different (P < 0.05).
additive effect on progesterone synthesis was achieved by a combination of pregnenolone with either A23187 or MIX (P < 0.01), whereas maximal stimulation (8-fold) (P < 0.01) was achieved by combination of all three additives.

**DISCUSSION**

Our studies demonstrate that dispersed bovine fetal cotyledon and maternal caruncle cells collected at days 120–283 of gestation produce progesterone in vitro. The ability of fetal cotyledons to secrete progesterone was evident in early as well as late pregnancy. However, appreciable progesterone synthesis by the maternal caruncles occurred only after 100–120 days of gestation. The production of progesterone by placental tissue may be important in the maintenance of gestation, as this steroid may act locally upon the uterus. The corpus luteum of pregnancy is generally assumed to be the primary source of progesterone during pregnancy in the cow, but recent studies on the function of bovine luteal cells from corpora lutea of pregnancy in vitro argue against a key role for this gland in late pregnancy as the cells have a markedly diminished capacity to synthesize progesterone in the basal state and to respond to LH (15). Moreover, the ovaries can be removed late in gestation in the cow or the corpora lutea can be prematurely regressed by exogenous prostaglandins without induction of abortion (3, 16). These findings point to a significant nonovarian source of progestins during gestation, which may well be the placenta.

The stimulatory effect of MIX on progesterone production by the bovine placental cells could be related to three basic actions of this compound: (i) the increased accumulation of cyclic nucleotides as a result of inhibition of phosphodiesterase (17); (ii) the translocation of intracellular calcium (18); and (iii) the blockade of receptors for adenosine (19). Since neither LH, 8-Br-cAMP, 8-Br-cGMP, chola toxin, or isoproterenol had effects on progesterone synthesis by itself nor did it potentiate the effects of LH, it is likely that MIX acted through translocation of intracellular calcium or through its effects on adenosine binding. The lack of responsiveness of the placental cells to adenosine supports the former concept. Furthermore, the calcium ionophore A23187 induced a 2-fold increase in progesterone production by the treated cells and had an additive effect when used in combination with MIX. Incubation of the cells in a calcium-free medium or in the presence of a calcium antagonist (TMB-8) reduced progesterone formation.

These findings indicate that translocations of intracellular calcium or influx of extracellular calcium stimulate progesterone production by placental cells. This action of calcium appears to be independent of cyclic nucleotides. Calcium ions have been implicated in the steroid biosynthetic processes in testes, adrenal glands, and ovaries (20–22). However, in all of these tissues cAMP is thought to be the primary intracellular regulator of hormone production. cAMP levels rapidly increase when ACTH or gonadotropins act upon the adrenal cortex or gonads, respectively, and cAMP analogues are potent stimulators of steroid hormone production by these tissues (23). The bovine placenta appears to differ from these other steroidogenic tissues and may resemble the zona glomerulosa of the adrenal cortex, a tissue in which calcium rather than cyclic nucleotides seems to be the primary intracellular signal increasing aldosterone synthesis (24–26).

It is not yet known whether a similar control process is used in placental steroidogenesis in other species. There have been reports that human placental progesterone synthesis is stimulated by human chorionic gonadotropin (27), presumably by a cAMP-mediated mechanism (28). However, the extent of stimulated production is small, leaving open the possibility of a significant role for calcium. It is interesting that steroidogenesis in the large luteal cells is not regulated by increased levels of intracellular cAMP both in ovine (29) and bovine (30) tissues.

Although the exact locus of calcium action in the steroidogenic process is not yet known, it is likely that it may influence the delivery or utilization of cholesterol by the mitochondria (31, 32). In addition, calcium may affect the conversion of pregnenolone to progesterone by Δ5-3-hydroxy-dehydrogenase (Δ5-3-OH-SDH), as a combination of exogenous pregnenolone and MIX or A23187 increased progesterone secretion over that seen with exogenous pregnenolone alone. This step in the biosynthesis of steroids is generally not thought to be regulated, and our observations are, therefore, unique in this regard. The factors that regulate calcium metabolism in the placental cells are not known. Our findings would suggest, however, that some humoral factor acts upon placental tissue and enhances steroidogenesis through the intermediacy of calcium.

In conclusion, our findings reveal that the bovine placenta has a mechanism of control of steroidogenesis that is cyclic nucleotide independent and apparently calcium dependent.

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