Influence of A23187 and dibutyryl cyclic AMP on progestagen production by rat granulosa cells \textit{in vitro}

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Summary. The gonadotrophic regulation of progesterone production by rat granulosa cells was examined in a chemically-defined medium containing FSH, dibutyryl cyclic AMP ((Bu)$_2$cAMP) and the calcium ionophore, A23187. FSH and A23187 alone significantly enhanced the production of pregnenolone, progesterone and its metabolite, 20α-hydroxypregn-4-en-3-one (20α-OH-P) from endogenous substrate(s). Stimulation of progesterone production by A23187 was accompanied by an increase in 3β-hydroxysteroid dehydrogenase (3β-HSD) but not 20α-hydroxysteroid dehydrogenase (20α-HSD) activity, as attested by enhancement of the metabolism of exogenous pregnenolone to progesterone but not of progesterone to 20α-OH-P. In contrast, although (Bu)$_2$cAMP increased pregnenolone and progesterone production and the metabolism of exogenous progesterone to 20α-OH-P, it failed to stimulate the conversion of exogenous pregnenolone to progesterone. The increase in progesterone production and in the conversion of exogenous pregnenolone to progesterone by FSH and A23187 was concentration- and time-dependent. Whereas maximal stimulation of de-novo progesterone synthesis by FSH was evident by 6 h (earliest time examined), a significant increase in the conversion of exogenous pregnenolone to progesterone in the presence of FSH or the ionophore was not noted until 12 h of incubation. Although a small but significant increase in progesterone production was also noted as early as 6 h of incubation in the presence of the calcium ionophore, this was markedly smaller than that elicited by FSH.

We conclude that the calcium ionophore A23187 and (Bu)$_2$cAMP have similar as well as distinct effects on progesterone production in rat granulosa cells \textit{in vitro}. We suggest that, while cAMP may be involved in the more rapid control by gonadotrophin of the production of the steroid via increased synthesis of pregnenolone and/or its metabolism to 20α-OH-P, calcium may be important in the synthesis and metabolism of pregnenolone for the maintenance of steroidogenic capacity of the granulosa cells.

Keywords: calcium; cAMP; 3β-hydroxysteroid dehydrogenase; 20α-hydroxysteroid dehydrogenase; steroidogenesis; granulosa cell; gonadotrophin action; rat

Introduction

Studies on the mechanism of hormone action have demonstrated that cAMP and calcium play important roles as transducers of hormonal stimuli in effecting cellular responses (Rasmussen & Goodman, 1977; Borle, 1981). In the gonadotrophic regulation of ovarian steroidogenesis, both cAMP and calcium are involved in the control of progesterone synthesis (Marsh, 1976; Veldhuis &
Klase, 1982a, b; c; Carnegie & Tsang, 1983, 1984; Tsang & Carnegie, 1983, 1984; Veldhuis et al., 1984; Asem & Hertelendy, 1986). While cAMP is believed to be important in the regulation of mitochondrial cholesterol uptake and of the activities of various steroidogenic enzymes, including cholesterol esterase and cholesterol side-chain cleavage enzymes (Behrman & Armstrong, 1969; Marsh, 1976), the exact role of calcium in the gonadotrophic control of ovarian steroidogenesis remains to be determined.

In-vitro studies with rat granulosa cells have suggested that calcium is required for the gonadotrophic control of cellular cAMP levels (Tsang & Carnegie, 1983; Eckstein et al., 1986). Incubation of granulosa cells with lanthanum, a calcium channel blocker, or an inhibitor of calmodulin, markedly reduced LH- and FSH-stimulated production of both cAMP and progesterone. Investigations with the calcium chelator, EGTA, have demonstrated that the calcium-dependence of progesterone production can be dissociated from the requirement for calcium in the synthesis of the cyclic nucleotide, suggesting that, in addition to regulating cAMP metabolism, calcium may be important at some step(s) on the steroidogenic pathway distal to the cAMP cascade (Carnegie & Tsang, 1983; Tsang & Carnegie, 1983).

In the present studies we have further examined the regulation of granulosa cell progesterone production by comparing the influence of the calcium ionophore, A23187, dibutyl cAMP [(Bu)2cAMP] and FSH in vitro on different steps in the enzymic pathway of progestagen synthesis.

Materials and Methods

Immature Sprague-Dawley rats (28 ± 1 days old) were injected intraperitoneally with 4 i.u. pregnant mares’ serum gonadotrophin (Equinex, Ayerst Labs., Inc., Montreal, Quebec, Canada) to induce follicular development. Follicles stimulated with this regimen were primarily at the antral stage when the animals were killed on the morning of Day 30 ± 1 (Fortune & Armstrong, 1977). Granulosa cells were released, by follicle puncture, into Eagle’s Minimal Essential Medium (MEM) containing NaHCO₃ (2.2 g/l), nonessential amino acids (0.1 mM), penicillin-streptomycin (50 000 U/l and 50 000 μg/l, respectively), and fungizone (625 μg/l) (all from Gibco Laboratories, Burlington, Ontario, Canada), and collected by centrifugation (180 g, 10 min). Removal of non-viable cells was achieved with a sequential trypsin-DNase treatment (Farookhi, 1982). Briefly, the granulosa cell suspension (10⁶ cells/ml) was incubated at 37°C with trypsin (50 μg/ml; Sigma Chemical Company, St Louis, MO, USA) for 1 min, excess soybean trypsin inhibitor (0.15 mg/ml; Sigma) was added and the suspension was then incubated with DNase 1 (25 μg/ml; Sigma) for 5 min. This procedure enriched the granulosa cell preparations to an initial value of 25-30% viable cells to a final level of 90-95%. Cells (3.5 × 10⁶/ml) were cultured for 6, 12 or 24 h, at 36°C in an atmosphere of 5% CO₂ and 95% air in MEM containing various agents [FSH (0-200 ng, NIMADD; of FSH-13/ml), A23187 (0-4 μg/ml; Sigma) or (Bu)₂cAMP (0-1 mM)]. To examine the influence of these agents on various steroidogenic steps, metabolism of exogenous steroid precursors was investigated in the absence of aminogluthethimide phosphate (AGP; 0-75 mM; CIBA-Geigy Corporation, Ardsley, NY, USA), an inhibitor of cholesterol side-chain cleavage (Kowal, 1969) or cyanoketone (0.25 μM; Winthrop Laboratories, Aurora, Ontario, Canada), an inhibitor of 3β-hydroxysteroid dehydrogenase (Goldman et al., 1965).

Medium collected at the end of the culture period was extracted twice with diethyl ether and the extract analysed for pregnenolone, progesterone, 17α-hydroxyprogesterone (17α-OH-P) and 20α-hydroxyprogren-4-ene-3-one (20α-OH-P) by specific radioimmunoassays (Orczyk et al., 1979; Inaba et al., 1979). The antisera for the progesterone and 20α-OH-P assays showed negligible cross-reactivity (0-1%) with other progestagens, androgens and oestrogens, with the exception of the latter which cross-reacted significantly with 20β-hydroxyprogren-4-ene-3-one (8-7%). The antisera for the pregnenolone assay exhibited significant cross-reactivity with 17α-hydroxyprogrenolone (12-7%), 5-pregnene-3β,20α-diol (25-0%), 5-pregnene-3β,20β-diol (8-3%), dehydroepiandrosterone (8-7%) and 5-androstone-3β,17β-diol (5-6%). Cyanoketone cross-reacted slightly but insignificantly with antisera for progesterone (0-001%) and 20α-OH-P (0-02%) while AGP and A23187 had negligible cross-reactivities with antisera for pregnenolone, progesterone and 20α-OH-P (< 0-001%). The 17α-OH-P antisera cross-reacted with pregnenin-4-ene-20β-ol-3-one (1-2%) but < 0-1% with progesterone, testosterone, oestradiol-17β, pregnenin-4-ene-20α-ol-3-one, 5α-pregnene-3,20-dione and 5α-pregnanediene-3α-ol-20-one.

Results were assessed statistically by analysis of variance. Significance of difference between groups was determined with Duncan’s new multiple range test or Student’s t test.

Results

The influence of FSH and the calcium ionophore A23187 on progesterone, 17α-OH-P and 20α-OH-P production by rat granulosa cells in vitro is summarized in Table 1. Whereas both FSH
(75 ng/ml) and A23187 (1 µg/ml) significantly enhanced \( P < 0.001 \) progesterone and 20α-OH-P production (Table 1; Fig. 1), synthesis of 17α-OH-P by granulosa cells was unaltered by either FSH or A23187 \( P > 0.05; \text{Table 1} \). There was a tendency for the ionophore to be slightly more effective than FSH in increasing 20α-OH-P production from endogenous substrates but this difference was not statistically significant \( P > 0.05; \text{Table 1} \). The effect of A23187 on progesterone production appeared to be due to its stimulatory action on pregnenolone synthesis and on the metabolism of the latter to progesterone (Fig. 1). A23187 (0.25–0.5 µg/ml) significantly increased pregnenolone accumulation during a 24 h incubation period \( P < 0.01 \) but 2 µg/ml was inhibitory \( P < 0.05 \). Like FSH, which stimulated the conversion of exogenous pregnenolone to progesterone in the presence of AGP (to inhibit endogenous pregnenolone formation) (Fig. 2), the calcium ionophore A23187 also increased the accumulation of progesterone from exogenous pregnenolone in a concentration-dependent manner (Fig. 1).

### Table 1. Influence of FSH and A23187 on progestagen production by rat granulosa cells during a 24-h culture period

<table>
<thead>
<tr>
<th>Groups</th>
<th>Steroid produced (pg/10⁵ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Progesterone</td>
</tr>
<tr>
<td>Control</td>
<td>44.8 ± 13</td>
</tr>
<tr>
<td>FSH (75 ng/ml)</td>
<td>554 ± 118*</td>
</tr>
<tr>
<td>A23187 (1 µg/ml)</td>
<td>223 ± 45*</td>
</tr>
</tbody>
</table>

Values are mean ± s.e.; \( n = 20; 5 \) experiments.

* \( P < 0.01 \) vs control in the same column.

![Fig. 1](image_url)  
Fig. 1. Influence of A23187 on production of progesterone (■), 20α-OH-P (▲) and pregnenolone (○) and on conversion of exogenous pregnenolone \( (P_5; 0.1 \mu m) \) to progesterone \( (P_4) \) (in the presence of 0.75 mM-AGP) (●) by rat granulosa cells incubated for 24 h in the presence of various concentrations of the ionophore \( (0–4 \mu g/ml) \). Values are mean ± s.e. \( (n = 12; 3 \) experiments).
Figure 2. Concentration-dependent stimulation by FSH of rat granulosa cell conversion of exogenous pregnenolone (P₅; 0-1 µM) to progesterone (P₄) during a 24-h culture period. Medium contained AGP (0-75 mM) to inhibit production of endogenous pregnenolone. Values are the means ± s.e. (n = 12; 3 experiments).

Figure 3 illustrates a time-course study of the regulation of granulosa cells progesterone production by FSH (75 ng/ml) and A23187 (1 µg/ml) in vitro. Addition of FSH to the incubation medium resulted in a stimulation (400%) of de-novo progesterone production within 6 h (earliest time examined). A small but significant increase in progesterone production was also noted in granulosa cells incubated in the presence of the calcium ionophore for 6 h. This latter response, however, was minimal when compared with that observed in the presence of FSH. In the presence of AGP (0-75 mM), the conversion of exogenous pregnenolone to progesterone elicited by FSH or A23187 occurred only after 12 h (Fig. 3). At a concentration which markedly stimulated pregnenolone and progesterone production (0-1 mM), (Bu)₂cAMP, an active analogue of cAMP, had no significant effect (P > 0.05) on the synthesis of progesterone from exogenous pregnenolone (Table 2). Moreover, while FSH (75 ng/ml), (Bu)₂cAMP (0-1 mM) and cholera toxin (10 ng/ml) significantly increased the metabolism of progesterone to 20α-OH-P by 250, 300 and 500% respectively (Table 3), A23187, at a concentration (1 µg/ml) which stimulated progesterone production as well as the metabolism of pregnenolone to progesterone, was ineffective (P > 0.05).

Figure 4 illustrates the possible dependence on protein synthesis stimulation by FSH and A23187 in granulosa cell progesterone and 20α-OH-P production in vitro. Addition of cycloheximide, an inhibitor or protein synthesis, to the culture medium (28 ng/ml) markedly reduced the production of progesterone and 20α-OH-P elicited by FSH and A23187. Maximal inhibition of progesterone production occurred at an inhibitor concentration of 28 ng/ml; a higher concentration further decreased (by 80-90%) production of 20α-OH-P (Fig. 4).

Discussion

Results from our earlier studies have suggested that calcium plays an important regulatory role in rat granulosa cell progesterone synthesis at point(s) in the steroidogenic pathway distal to the cAMP cascade (Carnegie & Tsang, 1984; Tsang & Carnegie, 1984). Although the regulation of cholesterol availability by cholesterol esterase (Behrman & Armstrong, 1969) and mitochondrial uptake (Marsh, 1976), cholesterol side-chain cleavage enzyme cytochrome P-450 levels (Toaff et
Fig. 3. Time course of progesterone production (—) and conversion of exogenous pregnenolone (P₅, 0·1 µM) to progesterone (P₄) in the presence of AGP (0·75 mM) (——) by rat granulosa cells incubated in the absence of (○) or presence of FSH (75 ng/ml; ●) or A23187 (1 µg/ml; ■). Progesterone production and conversion of pregnenolone to progesterone at 0, 6, 12 and 24 h in the control groups were 1·2, 10·1, 21·5, 35·9 and 1·9, 20·0, 36·3, 50·7 pg/10⁵ cells, respectively. Values are mean ± s.e. (n = 16; 4 experiments).

Table 2. Influence of (Bu)₂cAMP on pregnenolone and progesterone production by rat granulosa cells cultured for 24 h

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Treatment</th>
<th>Steroid produced (pg/10⁵ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Pregnenolone</td>
</tr>
<tr>
<td>A</td>
<td>None</td>
<td>25·7 ± 4·0</td>
</tr>
<tr>
<td></td>
<td>(Bu)₂cAMP (0·1 mM)</td>
<td>83·3 ± 5·3*</td>
</tr>
<tr>
<td>B</td>
<td>AGP (0·75 mM) + pregnenolone</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>(0·1 µM)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AGP (0·75 mM) + pregnenolone</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>(0·1 µM) + (Bu)₂cAMP (0·1 mM)</td>
<td></td>
</tr>
</tbody>
</table>

Values are mean ± s.e.; n = 8; 2 experiments.
*P < 0·01 vs no treatment in same column.

Calcium, cAMP and rat ovarian steroidogenesis

...al., 1983) and 3β-HSD activity (Jones & Hsueh, 1982) are under the control of gonadotrophic hormones, the exact site(s) on the steroidogenic pathway which are calcium dependent remains to be defined. Results from the present investigation have demonstrated that A23187, like FSH, significantly stimulated granulosa cell production of pregnenolone, progesterone and 20α-OH-P. Calcium is known to stimulate pregnenolone production in testicular (Janszen et al., 1976) and adrenal (Farese & Prudente, 1978) mitochondrial preparations and to increase the rate of
Table 3. Effect of A23187, FSH, (Bu)$_2$cAMP and cholera toxin on the conversion of exogenous progesterone to 20α-OH-P by rat granulosa cells during a 24-h period

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Treatment</th>
<th>20α-OH-P produced (pg/10⁵ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Cyanoketone (CKT 25 μM)</td>
<td>431 ± 35</td>
</tr>
<tr>
<td></td>
<td>CKT + progesterone (0.1 μM)</td>
<td>753 ± 26</td>
</tr>
<tr>
<td></td>
<td>CKT + progesterone + A23187 (1 μg/ml)</td>
<td>753 ± 23</td>
</tr>
<tr>
<td></td>
<td>CKT + progesterone + FSH (75 ng/ml)</td>
<td>1928 ± 86*</td>
</tr>
<tr>
<td>B</td>
<td>CKT + progesterone</td>
<td>275 ± 38</td>
</tr>
<tr>
<td></td>
<td>CKT + progesterone + (Bu)$_2$cAMP (0.1 μM)</td>
<td>752 ± 76*</td>
</tr>
<tr>
<td></td>
<td>CKT + progesterone + cholera toxin (10 ng/ml)</td>
<td>1296 ± 178*</td>
</tr>
</tbody>
</table>

Values are mean ± s.e.; n = 8; 2 experiments.

*P < 0.01 vs CKT + progesterone in same experiment.

Fig. 4. Effects of cycloheximide on FSH- (75 ng/ml; △, ▲) and A23187 (1 μg/ml; ○, ●)-stimulated progesterone (-----) and 20α-OH-P (-----) production by granulosa cells during 24 h. In the absence of cycloheximide, production of progesterone and 20α-OH-P was 50.3 and 193 pg/10⁵ cells (control), 625 and 1467 pg/10⁵ cells (A23187 group). Values are mean ± s.e. (n = 8; 2 experiments).
side-chain cleavage of labelled cholesterol in testicular tissue (Drosdowsky et al., 1965). Moreover, incubation of adrenal and testicular cells with calcium and calmodulin entrapped in liposomes resulted in a significant increase in mitochondrial cholesterol accumulation (Hall et al., 1981a, b). Whether calcium is important in controlling any of the above steroidogenic processes in the granulosa cell awaits further investigations.

A significant finding of the present studies involves the steroidogenic step catalysed by the 3ß-HSD. Our results with respect to the stimulatory effects of FSH and A23187 on conversion of exogenous pregnenolone to progesterone were rather unexpected, since this step is known not to be regulated by gonadotrophins (Asem et al., 1984; Shemesh et al., 1984) and to be independent of the influence of calcium in pig (Veldhuis & Klase, 1982a) and chicken (Asem & Hertelendy, 1986) granulosa cells. However, these observations stemmed from studies done in short-term incubation (3–4 h) whereas ours were made after 24 h of culture. Nevertheless, our observations are in keeping with the reported stimulatory action of gonadotrophins on 3ß-HSD in rat granulosa cells in long-term culture (Dorrington & Armstrong, 1979; Zeleznik, 1979; Jones & Hsueh, 1982; Jones et al., 1983). Moreover, the report of Shemesh et al. (1984) that A23187 and 3-isobutyl-1-methylxanthine, both known to raise intracellular free calcium levels albeit by different mechanisms, stimulated exogenous pregnenolone conversion to progesterone in bovine placenomes gives support to our findings. Our findings extend these observations and provide evidence consistent with an involvement of calcium in the regulation of this enzyme in the granulosa cell.

Although calcium is implicated in the action of A23187 on the 3ß-HSD catalysed step, other possible effects of the ionophore cannot be ruled out. A23187 may facilitate inositol-1,4,5-triphosphate and diacylglycerol production, with enhanced progesterone production resulting from increases in intracellular calcium and protein kinase C activation, respectively (Dimino et al., 1987; Wang & Leung, 1987). Veldhuis & Klase (1982b) showed that higher concentration of A23187 (10 µg/ml) significantly inhibited LH-stimulated progesterone production, depleted cell calcium stores and inhibited protein synthesis in pig granulosa cells. Although A23187 may have non-specific effects on granulosa cell function, such deleterious actions are observed mainly at excessively high concentrations (>2 µg/ml). Our findings that chelation of extracellular Ca²⁺ with EGTA (≥2.7 mM) suppresses the A23187 (1 µg/ml)-induced progesterone production (data not shown) lends support to the notion that the effect of the ionophore noted in the present studies may be calcium dependent.

The increase in net progesterone production by low concentration of A23187 (<1 µg/ml) did not appear to be a result of decreased conversion of progesterone to 20α-OH-P, as production of the latter steroid from exogenous progesterone was not affected by the ionophore. This conclusion was also supported by our observation that FSH, (Bu),cAMP and cholera toxin but not A23187 significantly increased metabolism of exogenous progesterone to 20α-OH-P. Our observation that A23187 is as effective as FSH in increasing 20α-OH-P production from endogenous substrate suggests that the 20α-HSD step is not rate-limiting and that the increased secretion of the steroid reflects increased substrate (progesterone) availability resulting from FSH or A23187 stimulation. It should, however, be noted that whereas A23187 significantly increased granulosa cell progesterone production from endogenous and exogenous substrate in a concentration-dependent manner (1–4 µg/ml), 20α-OH-P production from endogenous sterol was significantly enhanced with increasing concentration of the ionophore only up to 1 µg/ml. These findings suggest a possible non-specific inhibitory action of A23187 on 20α-HSD activity at high concentration and necessitate caution in the interpretation of the findings obtained under the latter conditions. Our findings are in good agreement with the earlier observation that ovarian 20α-HSD is under the control of gonadotrophin (Eckstein & Nimrod, 1979; Jones & Hsueh, 1981) and further suggests that this regulation may involve a cAMP-mediated mechanism.

Previous studies with testicular and adrenal tissues have demonstrated a requirement for calcium in the synthesis of proteins necessary for steroid hormone production (Hall & Eik-Nes, 1962; Farese, 1971a, b). Whereas dependence on protein synthesis in LH-stimulated steroid
production has been demonstrated in the ovary (Hermier et al., 1971; Arthur & Boyd, 1974; Toaff et al., 1979; Strauss et al., 1981), a role for calcium in this process has not been established. Results from the present investigations have shown that cycloheximide markedly inhibited granulosa cell progesterone and 20α-OH-P production elicited by FSH in vitro. While FSH is known to stimulate progesterone biosynthesis via cAMP, it is possible that a significant effect of the gonadotrophin may be mediated by a calcium-dependent system involving the synthesis of new protein. This idea is also consistent with the observed lag time in the stimulation of progesterone production by A23187 and on the conversion of exogenous pregnenolone to progesterone by FSH or the ionophore. Caution should, however, be exercised in the interpretation of the findings as they are only suggestive of a requirement for protein synthesis and the inhibitor may have non-specific actions. Moreover, whether the protein(s) presumably synthesized is involved in the intracellular transport of steroid precursors and intermediates, components of the cholesterol side-chain cleavage enzyme or of the 3β-HSD systems or modulators thereof remains to be determined.

Our findings do not negate the fact that the mitochondrial cholesterol side-chain cleavage enzyme catalyses the rate-limiting step along the steroidogenic pathway, neither do they promote 3β-HSD as a rate-limiting enzyme. Rather this communication adds to the growing evidence that the 3β-HSD may be an important controlling step in hormone-induced steroidogenesis, depending on experimental conditions as well as type and/or source of steroidogenic tissue.

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