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CONTROL OF STEROIDOGENESIS IN THE PREOVULATORY RAT FOLLICLE

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INTRODUCTION

The formation of steroids by cultured rat Graafian follicles explanted before the LH–FSH surge on the day of proestrus was first studied by Tsafriri and co-workers[1, 2]. They found that addition of LH *in vitro* resulted in an initial overall stimulation of steroidogenesis followed, after 4–6 h, by a decrease in androgen and oestrogen formation. In contrast progesterone formation was gradually increased. The involvement of the two main cell types which constitute the follicle, theca and granulosa cells, in this shift in follicular steroidogenic pattern prior to ovulation has recently been studied in our group.

It is now widely accepted that the main site for the stimulatory action of LH is the conversion of cholesterol to pregnenolone and, furthermore, that this effect is mediated by cyclic AMP [3]. The hypothesis of a co-operative function of theca- and granulosa cells in the production of follicular steroids is also more accepted although many of the details of this cell co-operation are still unknown [4]. At present little is known about the site(s) of inhibition leading to decreased secretion of oestrogens and androgens shortly after the initial stimulation. The possible involvement of cyclic AMP in this late effect of LH is still an open question.

Some illustrations from our long-term programme concerning these problems will be described. Changes in steroidogenesis and in cyclic AMP levels in the preovulatory follicle as well as in isolated follicular cells have been measured in relation to the preovulatory LH–FSH surge in a rat model where preovulatory follicles have been produced in the immature rat by the injection of a small dose of pregnant mare serum gonadotrophin (PMSG).

MATERIALS AND METHODS

Immature Sprague Dawley rats maintained under standardized conditions (light 05.00–19.00 h; 24°C) were injected subcutaneously (s.c.) with 8 IU PMSG on the morning of day 28 of age. This treatment resulted in the ovulation of 12 ± 2 ova between 02.00–05.00 h on day 31 [5] preceded by an endogenous LH–FSH surge between 15.00–18.00 on day 30. Ovarian follicles were isolated on day 30 either between 10.00–12.00, i.e. before the endogenous LH–FSH surge ("morning follicles"), or between 19.00–21.00, i.e. some hours after the endogenous LH–FSH surge ("evening follicles"). Eight to ten large follicles considered to be preovulatory were isolated from each rat by microdissection under the stereomicroscope. The follicles were either incubated directly or further mechanically separated into granulosa and theca cells as has been described in detail elsewhere [6]. The follicles and the isolated cells were incubated for 1–4 h in a modified Krebs bicarbonate buffer containing glucose (5.5 mM) and bovine serum albumin (1%). After incubation aliquots of the medium were rapidly withdrawn and stored at −80°C until analysed with radioimmunoassays for steroids [7]. Cyclic AMP was determined by the protein binding technique described by Gilman[8]. When whole preovulatory follicles were incubated, the cyclic AMP content was determined separately in tissue and incubation medium, whereas in experiments on isolated granulosa cells the content of cyclic AMP was determined in tissue plus medium.

RESULTS

Steroidogenesis

Follicles. Whole preovulatory follicles extirpated before the endogenous gonadotrophin surge ("morning follicles") secreted predominantly oestradiol and androstenedione and very low amounts of progesterone. Addition of LH *in vitro* increased the secretion of all three steroids (Fig. 1) [7]. "Evening follicles", removed after the endogenous gonadotrophin surge, formed small amounts of androstenedione and oestradiol when incubated in hormone-free medium, while progesterone secretion was markedly enhanced compared to the situation before the gonadotrophin surge. Addition of LH to "evening follicles" had no effect on androstenedione and oestradiol secretion, while the hormone was able to enhance further the formation of progesterone. Figure 1 also illustrates that steroid secretion by follicles removed in the evening from rats where the endogenous gonadotrophin surge was blocked by Nembutal was similar to that of "morning follicles" both in absence and presence of LH *in vitro*.

Addition of testosterone to isolated "evening follicles" markedly enhanced oestradiol secretion, while addition of dihydrotestosterone or 17-OH-progesterone had no effect (Fig. 2) [9]. Figure 3 illustrates that

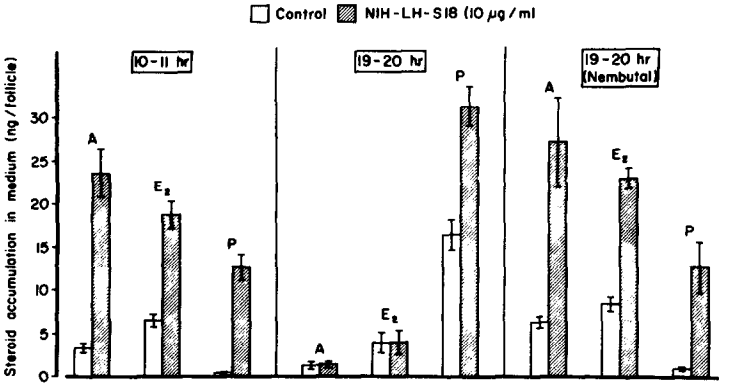

Fig. 1. Steroid formation by isolated preovulatory follicles incubated in the presence or absence of LH (10 μg/ml). Medium content of androstenedione (A), 17β-oestradiol (E_2) and progesterone (P) after 4 h incubation. Three groups of follicles were explanted at the times indicated on day 30, after the donors had been injected with PMSG on day 28. The 10–11 h group was from rats prior to the LH surge; the 19–20 h group, from rats after the endogenous LH surge; and the last group, from rats given Nembutal 4 to 5 h earlier. Each bar represents mean ± S.E. of 4–5 determinations [7].

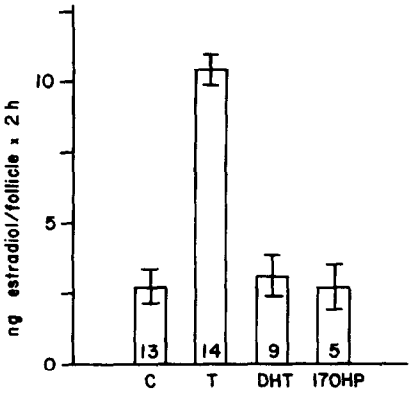

Fig. 2. Secretion of 17β-oestradiol isolated preovulatory follicles extirpated between 19.00–22.00 h on day 30 and incubated in plain medium (C) or medium supplied with 1 μg/ml of testosterone (T), 5α-dihydrotestosterone (DHT) or 17α-OH-progesterone (17-OH-P) for 2 h. Mean ± SE. Number of determinations indicated on the bars [9].

addition of testosterone did not incease progesterone production under any of the experimental conditions used.

Isolated follicular cells. Steroid formation by isolated granulosa and theca cells, respectively, and as a comparison, by whole follicles from the same rats are illustrated in Fig. 4 [10]. Addition of LH did not stimulate oestradiol formation by granulosa or theca cells isolated from "morning follicles" although progesterone formation by the granulosa cells and androstenedione formation by the theca cells were stimulated by this gonadotrophic hormone. Addition of testosterone increased oestradiol formation in the granulosa cells but not in the theca cells.

Granulosa and theca cells removed 3–5 h after the gonadotrophin surge, i.e. from "evening follicles", produced high amounts of progesterone while oestradiol and androstenedione formation was extremely low in both cell types. Granulosa and theca cells from "even-

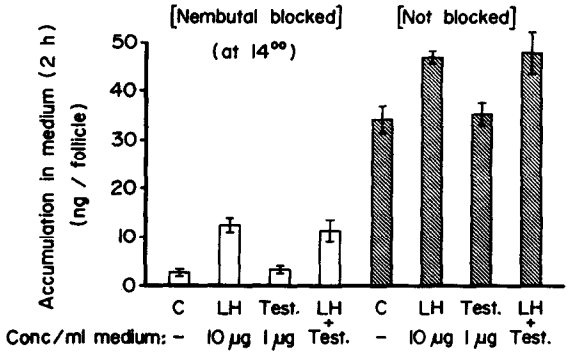

Fig. 3. Secretion of progesterone by isolated preovulatory follicles removed between 19.00–22.00 on day 30, and incubated in plain medium (C) or medium supplied with testosterone and/or LH for 2 h. One group of rats had received an i.p. injection of Nembutal (35 mg/kg body weight) at 14.00 h on day 30 (open bars) to block the endogenous gonadotrophin surge. The other group of rats was injected with saline at the same time (hatched bars). The effect of LH *in vitro* was significant ($P < 0.01$) both after Nembutal and saline treatment whereas there was no significant effects of testosterone.

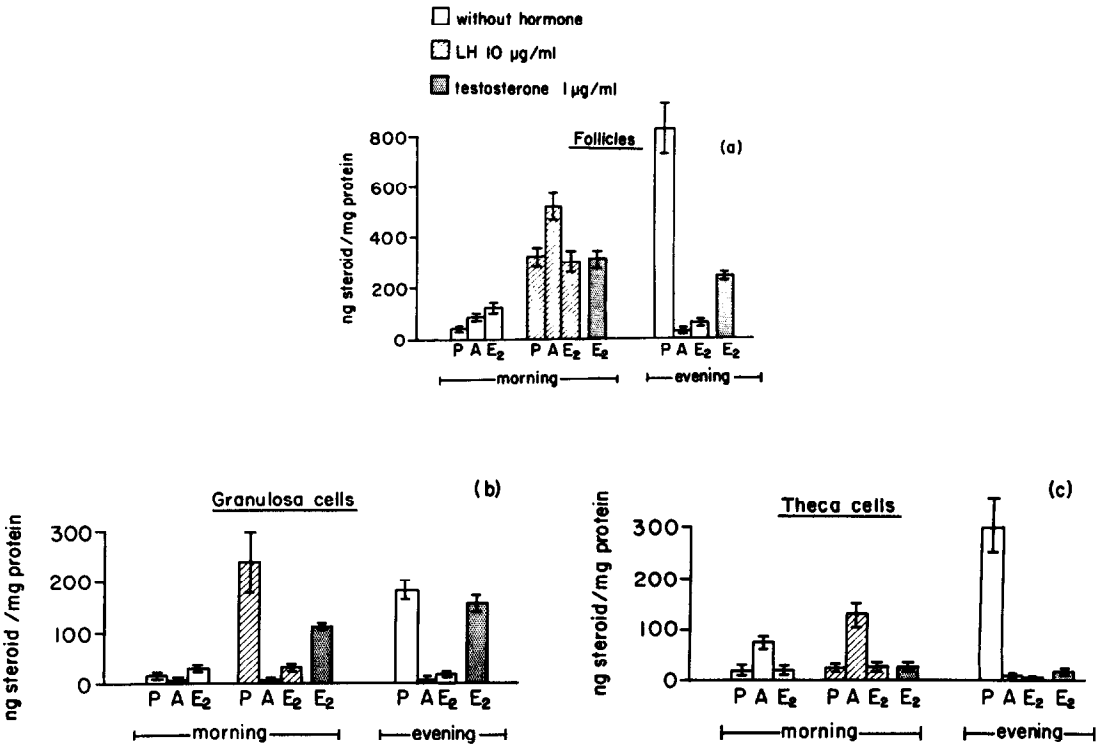

Fig. 4. Formation of steroids *in vitro* by intact preovulatory follicles (a), isolated granulosa cells (b) and isolated theca cells (c). The samples were isolated either 10.00–14.00 h (morning) or 20.00–24.00 h (evening) on day 30 and incubated for 2 h in hormone free medium (open bars), medium containing LH (10 μg/ml, hatched bars) or testosterone (1 μg/ml, dotted bars). The accumulation of progesterone (P), androstenedione (A) and estradiol (E_2) in the incubation medium was determined by RIA. The accumulation of A in the medium of granulosa cells isolated in the evening was below detection limits (<0.2 ng/ml) [10].

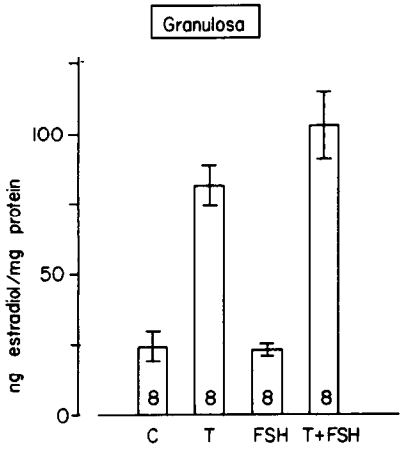

Fig. 5. Formation of 17β-oestradiol (E_2) by isolated granulosa cells incubated for 2 h either in plain medium (C) or in the presence of FSH 10 μg/ml, testosterone (T) 1 μg/ml, or a combination of FSH and testosterone. The cells were isolated 10.00–14.00 h (morning) on day 30, *i.e.* 2 days after PMSG. The accumulation of oestradiol in the incubation medium is given as mean ± S.E. of 8 determinations (equal to number of donor rats) [10].

ing follicles" increased their formation of oestradiol in presence of testosterone. The capacity of the theca cells to form oestradiol however, was low.

Addition of FSH together with testosterone to granulosa cells did not significantly increase the formation of oestradiol (Fig. 5) [10]. Conversion of 17-OH-progesterone to androstenedione in theca cells was seen only when the theca cells were removed from "morning follicles" (Fig. 6) [10].

Cyclic AMP formation

It is well established that LH *in vitro* markedly stimulates cyclic AMP production by isolated preovulatory follicles removed before the gonadotrophin surge [11, 12]. This effect is shown in Fig. 7 [12], which also illustrates that PGE_2 stimulates the formation of cyclic AMP in the preovulatory follicle. A more delicate problem has been to establish whether there is an increase in the cyclic AMP content of the preovulatory follicle after the endogenous LH–FSH surge. A negative report first appeared in the literature [13]. It has since then been found in our laboratory [14, 15] that there is a moderate

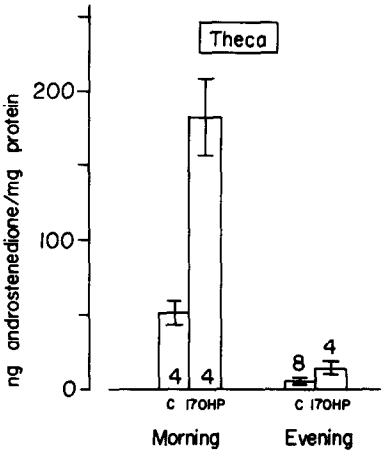

Fig. 6. Formation of androstenedione by isolated theca cells incubated for 2 h in plain medium or in the presence of 17-hydroxyprogesterone 1 μg/ml. The cells were isolated 10.00–14.00 h (morning) or 20.00–24.00 h (evening) on day 30. The accumulation of androstenedione in the incubation medium is given as mean ± S.E. The number of determinations in each group (equal to the number of donor rats) indicated on the bars [10].

(40–60%) and transient increase of cyclic AMP in the whole ovary during the afternoon of the day before ovulation (Table 1) [15].

In the intact preovulatory follicle there is also a moderate increase in tissue cyclic AMP content in the evening of the day before ovulation. This increase seems, however, to persist up to the time of ovulation [16]. Cyclic AMP levels in the granulosa cells of the preovulatory follicle have recently been measured [6, 17], and Fig. 8 demonstrates a preovulatory rise of cyclic AMP in the granulosa cells concomitant with and in the same order of magnitude as that of the whole ovary. Unlike the whole ovary, but like the whole follicle, the isolated granulosa cells showed no decrease in cyclic AMP levels close to ovulation.

An interesting phenomenon, recently described for various types of ovarian preparations, is the desensitization (or refractoriness) of the tissue to gonadotrophins induced by a previous stimulation with the same hormone [18]. A desensitization of the preovulatory follicle to LH after an endogenous gonadotrophin surge was first reported in the rabbit [19] where it was found that preovulatory follicles exhibited a markedly decreased sensitivity to LH *in vitro* in regard to steroid and cyclic AMP production during the period between the gonadotrophin surge and ovulation. A similar phenomenon has been observed in the preovulatory rat ovarian follicle after the endogenous LH–FSH surge. The follicular refractoriness in the rat seems, however, not to be complete but relative (Fig. 9) [16]. Under *in vitro* conditions it has been clearly shown that prolonged exposure of the preovulatory rat follicle *in vitro* to LH or FSH induced refractoriness to a subsequent challenge with fresh hormone, and this desensitization has been shown to be hormone specific, and concentration and time-dependent [18]. With the above mentioned facts as a background it was a surprise when it was found that granulosa cells removed from the preovulatory rat follicle after the gonadotrophin surge did not show desensitization to LH when their ability to produce cyclic AMP was tested *in vitro* [17, 20, 21]. These cells were, on the opposite, more sensitive to LH than granulosa cells removed before the gonadotrophin surge. The reason for this difference in reactivity between the whole preovulatory follicle and the isolated granulosa cells from such follicles is presently under investigation in our laboratory and some findings are illustrated in Figs. 10 and 11. Figure 10 shows that pretreatment of rats in the morning of day 30, i.e. before the gonadotrophin surge, with a

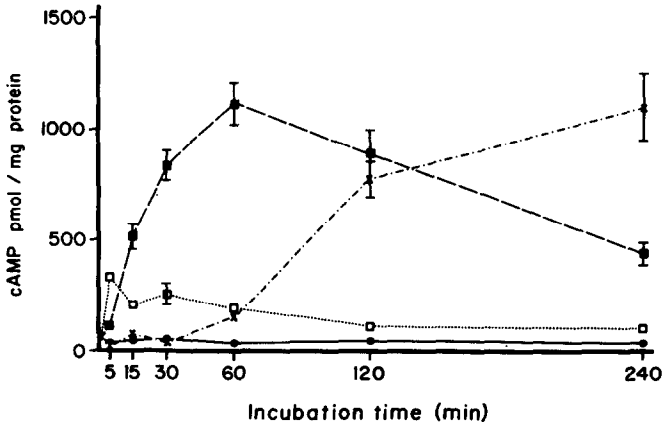

Fig. 7. Time course of cyclic AMP formation in isolated preovulatory follicles with and without hormones. The follicles were removed before the LH–FSH surge ("morning follicles"). After 30 min preincubation, isolated preovulatory follicles were incubated from 5–240 min, in plain medium (●——●), medium containing LH, 10 μg/ml (■– – –■), medium containing PGE_2, 10 μg/ml (□·····□) and *tissue* levels of cyclic AMP were measured. Also shown in the figure are the levels of cyclic AMP in the *medium* after LH addition (×—·—×) [12].

Table 1. Preovulatory changes (mean ± S.E.M.) in serum LH and ovarian cyclic AMP levels in immature rats treated with PMSG

Time (h)	LH (ng/ml)	Cyclic AMP (pmol/mg protein)
Day 30		
12.00–14.00	108 ± 4	31.5 ± 1.4
14.00–16.00	171 ± 42	33.6 ± 1.7
16.00–17.00	338 ± 72*	37.9 ± 2.8
17.00–18.00	305 ± 53*	39.8 ± 2.6*
18.00–19.00	484 ± 97*	43.9 ± 4.5*
19.00–20.00	—	42.7 ± 4.0*
21.00–23.00	216 ± 35*	36.0 ± 2.8
23.00–01.00		36.9 ± 1.6
Day 31		
02.00–04.00	111 ± 5	33.3 ± 2.4

* These values were significantly different (<0.05) from the corresponding ones at 12.00–14.00 h (analysis of variance) [15].

single i.p. dose of LH 2 h before sacrifice potentiated the subsequent *in vitro* effect of LH on cyclic AMP formation by the granulosa cells. Figure 11 [22] illustrates in similar experiments that addition of follicular fluid (approx. 1%) to the incubation medium nearly completely blocked the *in vitro* effect of LH on the isolated granulosa cells. This finding suggests the existence of a factor in the follicular fluid of the rat ovary that interacts with the stimulatory effect of LH on the granulosa cells. Such a factor might explain why the isolated whole follicle containing the follicular fluid shows refractoriness to LH stimulation after the gonadotrophin surge while the isolated granulosa cells still show a high sensitivity to LH.

DISCUSSION

A two-cell type model for the production of follicular oestrogens has been suggested from many experiments and the results described in this review are in favour of such a model, at least for the preovulatory follicle of the rat. Granulosa cells isolated from these preovulatory follicles seem to lack the enzymes necessary for the conversion of progesterone to androgens, and the capability of theca cells to aromatize androgens to oestrogens seems to be very limited. The theca cells thus are believed to produce the androgen substrate which is transported into the granulosa cells for aromatization to oestrogens. In all studies where ovarian cells have been enzymatically or mechanically separated before incubation the possibility of introducing experimental errors in the form of deprivation of specific co-factors, selective disturbances of vulnerable enzymes etc. must, however, be considered. For this reason a lack of capacity in one or several enzyme systems in an isolated cell system must be evaluated with the utmost criticism.

It was recently shown that isolated granulosa cells from immature or hypophysectomized rats did not aromatize testosterone to 17β-oestradiol unless FSH was also present [93]. A specific effect of FSH on the granulosa cell aromatase enzyme has therefore been suggested [4]. Our results illustrate (Fig. 4b) that granulosa cells isolated from the preovulatory follicle

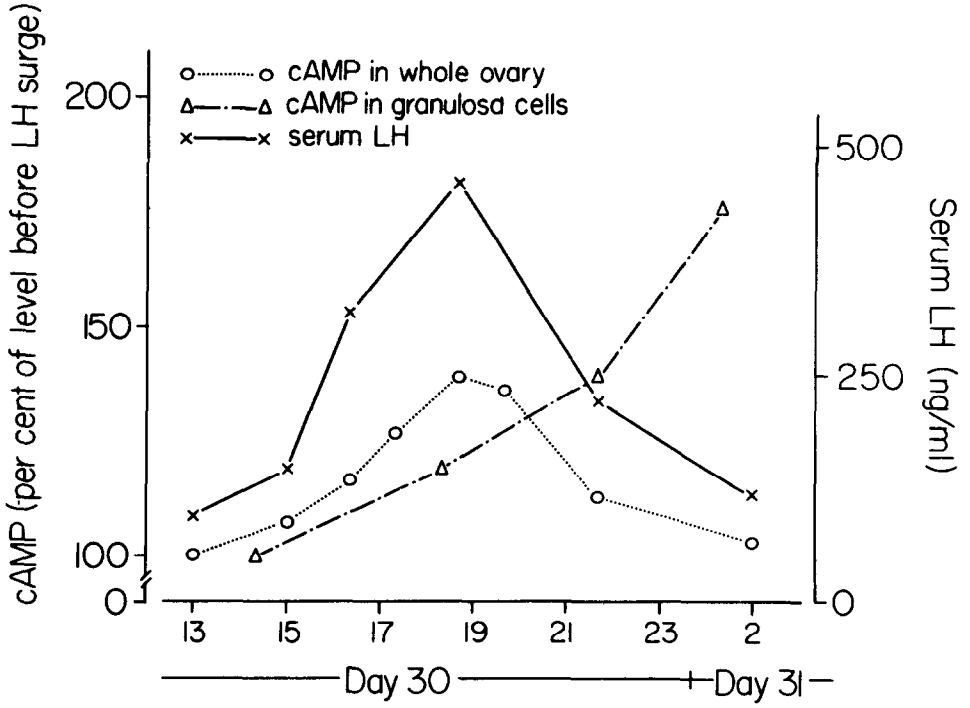

Fig. 8. Serum LH values and cAMP levels of whole ovaries and isolated granulosa cells in 30–31 day old rats injected with 8 IU PMSG on day 28 [15, 6].

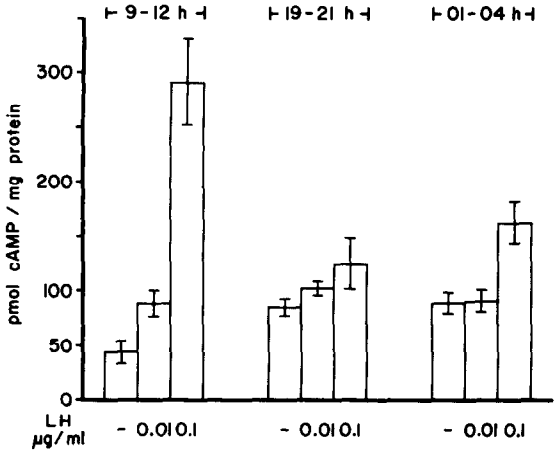

Fig. 9. Effects of different concentrations of LH on tissue cyclic AMP levels in pre-ovulatory rat ovarian follicles from PMSG treated immature rats. The three left bars represent follicles explanted into incubation medium before the endogenous LH-FSH peak (09.00–12.00 h on day 30), the three middle bars represent follicles explanted after the peak (19.00–21.00 h on day 30) and the three right bars represent follicles explanted just before ovulation (01.00–04.00 h on day 31). The follicles were incubated for 2 h in absence or presence of NIH-LH-B9 in the concentrations indicated. Note increased cyclic AMP levels in control groups and decreased sensitivity to LH *in vitro* after the LH-FSH peak [16].

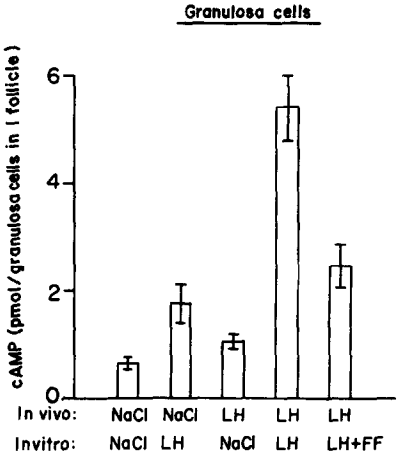

Fig. 11. Effects of LH plus follicular fluid *in vitro* on cAMP formation in isolated granulosa cells after *in vivo* injection of LH or saline. Experimental conditions the same as in Fig. 10 with the addition that one group of granulosa cells was incubated with LH plus follicular fluid. This fluid was taken from isolated preovulatory follicles which were punctured in chilled buffer. Thereafter the buffer, containing follicular fluid and granulosa cells, were centrifuged at high speed. The supernatant then was used as incubation medium, with or without addition of LH (1 µg/ml). The concentration of follicular fluid in the incubations was approx. 1% [22].

either before or after the LH–FSH surge have a high capacity to aromatize testosterone without the addition of FSH. This probably means that the FSH effect on granulosa cell aromatizing enzymes is initiated early in the follicular development.

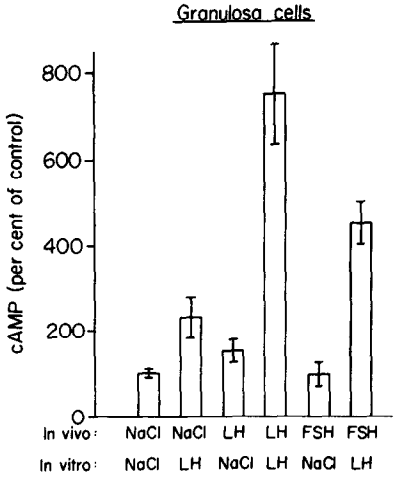

Fig. 10. Effects of LH *in vitro* on cyclic AMP formation in isolated granulosa cells after *in vivo* exposition (2 h) to LH, FSH or saline. The cells were isolated from preovulatory follicles removed before the endogenous LH-FSH peak (morning day 30). The rats were pretreated with a single i.p. injection of LH or FSH (10 µg/rat) or saline. Incubation time was 1 h with or without addition of LH (1 µg/ml) to the medium. Cyclic AMP was determined in cells plus medium [21].

The primary interaction of LH with its target cells is believed to involve specific saturable plasma membrane receptors. Granulosa cells of small and medium sized follicles have no or very few LH receptors while these receptors have developed in the granulosa cells of the preovulatory follicle [24, 25]. Theca cells of both small and large follicles have LH receptors. When the LH of the gonadotrophin surge reaches the ovary, this hormone will therefore probably bind to both theca and granulosa cells of the Graafian follicles. In experiments with isolated theca and granulosa cells, this LH receptor interaction leads to activation of adenylate cyclase with increased cellular levels of cyclic AMP in both cell types. This is also reflected in corresponding changes in the intact preovulatory follicle. As mentioned above this increase in cyclic AMP is currently believed to be the triggering mechanism for an increased rate of conversion of cholesterol to pregnenolone, and such an increased formation of pregnenolone occurs thus probably in both theca and granulosa cells. An interesting hypothesis is that the observed changes in the pattern of follicular steroidogenesis in the period between the LH–FSH surge and ovulation represent a "shut-down" of theca cell steroid secretion by a block in androgen formation, so that the progesterone production of both granulosa and theca cells becomes unmasked, which would represent the biochemical basis for luteinization. A difference in pattern of refractoriness to gonadotrophic stimulation between the two cell-types might act to enhance this differentiation.

In this context it should be remembered that the internal millieu for the granulosa cells before follicular rupture is the follicular fluid, which has been shown to contain a variety of regulatory peptides. One of these regulators has been termed a "luteinization inhibitor" [26, 27]. This factor was extracted from porcine follicular fluid and was shown to inhibit the HCG-stimulated rise of cyclic AMP as well as progesterone production by granulosa cells. A similar effect has recently been described with human follicular fluid, which was reported to inhibit the HCG-stimulated increase in isolated prepubertal mice ovaries [28, 29]. The inhibitory effect was most pronounced when the follicular fluid was taken from large preovulatory follicles. It is tempting to speculate that it is a similar inhibitor which acts in our system counteracting the stimulatory effect of LH on the granulosa cells. Removing the follicular fluid from the granulosa cells might then mimic the effects of follicular rupture and reveal the luteotrophic effect of LH.

These findings also point to the importance of keeping in mind the obviously unphysiological experimental situation which is inherent in every investigation involving isolated cell systems. The presentation in this review is an attempt to emphasize the importance of comparing results from isolated cell experiments with both the intact follicle and the whole ovary in the same species in order to make physiological interpretations of the results.

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