

Studies on the Mechanism of Luteinizing Hormone-Induced Desensitization of the Rabbit Follicular Adenylyl Cyclase System *in Vitro**

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ABSTRACT. *In vitro* studies were conducted to evaluate the possible mechanisms of LH-induced desensitization of the rabbit follicular adenylyl cyclase (AC) system. We tested the effects of cAMP, dibutyryl cAMP, and inhibitors of various cellular functions on LH-stimulated AC activity as well as the reversibility of AC desensitization. Refractoriness of the AC to LH was induced by a 1- or 2-h incubation of Graafian follicles with 10 $\mu\text{g/ml}$ LH. We found that the initial 60-min phase of AC desensitization to LH in Graafian follicles was not prevented by a 60-min preincubation of follicles with 11 μM puromycin, 30 μM cycloheximide, 8 μM actinomycin D, 5 $\mu\text{g/ml}$ cytochalasin B, 50 μM colchicine, or 1 or 10 mM trinitrophenol or by a 90-min preincubation of follicles with 50 μM colchicine. We evaluated the effects of cAMP and dibutyryl cAMP on LH-stimulated AC activity by incubating Graafian follicles with these nucleotides for 30-min to 4 h. While LH-stimulated AC activity was not significantly reduced

in follicles which had been incubated 30-min or 1-h with either nucleotide, 2 h incubations resulted in significant reductions in LH-stimulated AC activity, and 4-h incubations promoted a complete refractoriness of the LH-stimulable AC. cAMP also caused desensitization of the FSH-stimulable AC in 4-h incubations, but not in the 1-h incubations. Lastly, once the follicular AC was desensitized to LH, neither 5'-guanylyl imidodiphosphate nor ATP could reverse desensitization. These results indicate that AC desensitization in rabbit Graafian follicles is a biphasic event. The initial 60-min phase is not mediated by cAMP, RNA, or protein synthetic events, by energy-requiring events inhibited by trinitrophenol, or by microtubule- or microfilament-associated processes. A secondary phase occurs within 2-h and appears to be mediated, at least in part, by cAMP. (*Endocrinology* 109: 345, 1981)

THE PREOVULATORY surge of gonadotropins promotes a time-dependent desensitization *in vivo* of the LH-sensitive adenylyl cyclase (AC) to LH in Graafian follicles of rats and rabbits (1, 2). However, while a 50% drop in follicle LH-stimulated AC is demonstrable within 1-h of coitus or hCG injection in rabbits (1), a 50% decline in LH-stimulated AC is not detectable in proestrous rat follicles until they ovulate (2), some 12-h after the critical period of gonadotropic secretion. Studies in rat follicles reveal that LH-induced desensitization *in vitro* requires an actinomycin D-sensitive step (3). The much more rapid rate of desensitization in rabbit follicles both *in vivo* and *in vitro* (1) prompted our investigation of the effects of various inhibitors of RNA and protein synthesis, disruptors of the cytoskeletal system, and cAMP and dibutyryl cAMP as potential feedback regulators on LH-

responsive AC activity. We also investigated the reversibility of AC desensitization in Graafian follicles.

Results reveal that the initial 60- to 90-min phase of AC desensitization to LH in rabbit follicles does not require RNA or protein synthetic events or endocytotic microtubule- or microfilament-requiring processes, nor is it mediated by cAMP or dibutyryl cAMP. However, a feedback role for cAMP is suggested within 120 min by the ability of cAMP and its dibutyryl analog to cause a partial reduction in LH-stimulated AC activity. Additionally, neither ATP nor 5'-guanylyl imidodiphosphate [GMP-P(NH)P] promote resensitization of the desensitized Graafian follicle AC.

Materials and Methods

Materials

LH (NIH-LH-B8, NIH-LH-B9, NIH-LH-B10, and NIH-LH-S18) and FSH (NIH-FSH-S13) were gifts of the Pituitary Hormone Distribution Program, NIAMD, NIH (Bethesda, MD). LH was dissolved with 0.1 M NaCl to a concentration of 1 mg/ml and was diluted freshly for individual assays. Dibutyryl cAMP, cycloheximide, colchicine, and dimethylsulfoxide (DMSO) were obtained from Aldrich Chemical Co. (Milwaukee,

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WI). Puromycin hydrochloride, cytochalasin B, creatine phosphate, and creatine kinase were obtained from Calbiochem (San Diego, CA). [α - 32 P]ATP, [3 H]cAMP, L-[G- 3 H]amino acid mixture, and [3 H]uridine were obtained from New England Nuclear Corp. (Boston, MA). GMP-P(NH)P was obtained from ICN (Irvine, CA). Picric acid [2, 4, 6-trinitrophenol (TNP)] was obtained from Fisher Chemical Co. (Pittsburgh, PA) and was recrystallized four times before use. Actinomycin D was freshly diluted with 100% ethanol; puromycin, and colchicine were freshly diluted with Krebs-Ringer bicarbonate buffer (KRB); and cytochalasin B was freshly diluted with DMSO. The remaining biochemical reagents, including some batches of dibutyl cAMP, were purchased from Sigma Chemical Co., (St. Louis, MO).

Animals and dissections, distribution and incubation of follicles

Rabbits were maintained as previously described (1). Females which were neither pregnant nor pseudopregnant were considered to be in estrus. Rabbits were sacrificed by cervical dislocation between 1000-1200 h. Ovaries were removed and placed in 0.9% NaCl at room temperature. Follicles from each rabbit of greater than 1.0 mm in diameter (four or five per ovary) were dissected and distributed, each to a separate flask (20-ml glass scintillation vial) containing 2.5 ml KRB with 1 mg/ml glucose. Flasks with four or five follicles (each from a different rabbit) were incubated at 37 C for the indicated times in the presence of no further additives or with bovine serum albumin (BSA), LH (NIH-LH-S18), or the various substances shown in the legends to the figures and tables, each added in 10- or 20- μ l volumes. During incubation, each vial was gassed with a warmed and humidified mixture of 95% O₂ and 5% CO₂. After incubation, follicles were rinsed by a 15-min incubation at 37 C in KRB-glucose, vials were placed on ice, and follicles were popped and homogenized for AC assay determinations.

AC assay

Follicles were homogenized in 0.5 ml of a medium containing 27% (wt/wt) sucrose, 1 mM EDTA, and 10 mM Tris-HCl, pH 7.5, using 10 strokes with the tight pestle of an all-glass Dounce homogenizer, as previously described (1). The incubation for AC activity present in silk-screen-filtered homogenates was carried out in duplicate or triplicate in the presence of BSA (basal activity), LH (NIH-LH-B8, NIH-LH-B9, or NIH-LH-B10), FSH (NIH-FSH-S13), or NaF in a medium containing 3.0 mM [α - 32 P]ATP, 5 mM MgCl₂, 1 mM [3 H]cAMP, 1 mM EDTA, and a creatine kinase-creatine phosphate regenerating system, as previously described (1).

Protein was determined by the procedure of Lowry *et al.* (4) using crystalline BSA as standard. Statistical significance was evaluated with Student's *t* test.

Estimation of the inhibition of follicular RNA, DNA, and protein synthesis by inhibitors

Graafian follicles were distributed, as previously described, into flasks (six to eight follicles per flask) containing 1 ml KRB-glucose. At time zero, drugs were added in a 10- μ l volume such

that final concentrations were 10 μ M puromycin, 30 μ M cycloheximide, and 8 μ M actinomycin D. Incubations were performed at 37 C with continuous gassing (95% O₂-5% CO₂). Forty-five minutes after initiation of the incubation, [3 H]amino acid mixture (15 L-amino acids; 2 μ Ci in 10 μ l KRB) or [3 H]uridine (2 μ Ci in 10 μ l KRB) was added to the vials. Sixty minutes after the initiation of the incubation, 10 μ g/ml LH were added to the vials. Follicles were incubated for an additional 60-min (2-h total incubation time). Follicles were rinsed by 5-min incubation in 2.5 ml fresh KRB-glucose. Incubations were terminated by placing flasks on ice. The follicular RNA, DNA, and protein fractions were chemically separated in order to determine the incorporation of radiolabeled precursors into these fractions, according to the method of Shatkin (5). This technique involved homogenization of follicles, preincubated *in vitro* in the presence of radiolabeled amino acids and RNA precursors, in 5% perchloric acid (PCA). Acid-insoluble materials were pelleted and washed by centrifugation, and the pellet was defatted by treatment with cold ethanol, ethanol-ether, and then ether. RNA in this pellet was then hydrolyzed to ribonucleotides by overnight incubation at an alkaline pH, and the cellular DNA and protein were separated from the ribonucleotides with pH adjustment with PCA. DNA was hydrolyzed to its constituent bases in 5% PCA at 70 C. Protein precipitated from DNA bases upon cooling of the solution. RNA (6), DNA (7), and protein (4) contents were then determined in the respective fractions. Radioactivity in each fraction was determined by suspending portions of each fraction in 10 ml scintillation fluid (3a70B, Research Products Inc., Elk Grove Village, IL) and counting in a Packard Tri-Carb liquid scintillation counter (Packard, Downers Grove, IL). Appropriate backgrounds were determined and subtracted from experimental determinations.

Results

Effect of dibutyl cAMP and cAMP on follicular AC activity

The purpose of this study was to determine whether cAMP or its dibutyl derivative could promote desensitization of the follicular AC system to LH. Previous studies demonstrated that *in vitro* incubation of rabbit follicles in KRB-glucose for 1 or 2-h with LH mimicked the desensitization response of the AC to LH induced *in vivo* by hCG or LH (1). Using the same *in vitro* incubation procedure, follicles were incubated with BSA or LH or with various concentrations of dibutyl cAMP for 30-min or 1, 2, or 4-h.

We found that LH-stimulated AC activity was not effectively reduced by 30-min or 1-h incubations of follicles with 0.4-8 mM dibutyl cAMP, as shown in representative experiments in Fig. 1 and summarized in Table 1. Incubation of follicles with dibutyl cAMP for 2-h, however, promoted an apparent concentration-dependent decline in LH-stimulated AC activity (Fig. 1B), with a 52% decline ($P < 0.001$) induced by 8 mM dibutyl cAMP. Incubations for 4-h resulted in significantly reduced LH-stimulated AC activities ($P < 0.001$) with each

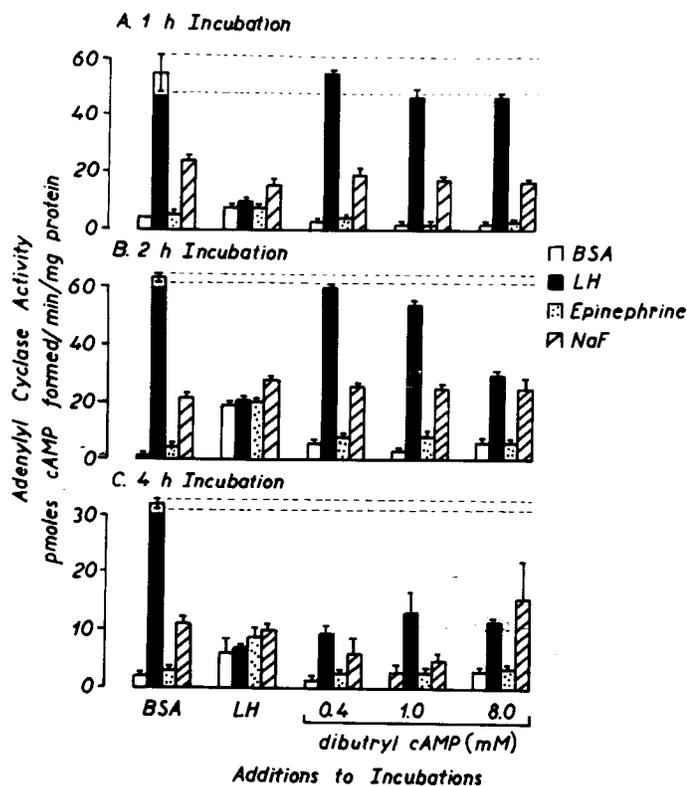


FIG. 1. Effect of *in vitro* exposure of rabbit Graafian follicles to dibutyryl cAMP on AC activity of follicular homogenates. Follicles from estrous rabbits (four to eight follicles per rabbit) were distributed into separate flasks. Flasks (four or five follicles per flask, each from a separate rabbit) were incubated for 1 h (A), 2 h (B), or 4 h (C) at 37 C in KRB-glucose containing BSA, 10 μ g/ml LH (NIH-LH-S18), or dibutyryl cAMP in the final concentrations indicated on the figures. After incubation and subsequent rinsing, follicles were popped and homogenized, and follicular homogenates were assayed for AC activity in the presence of BSA (\square), 10 μ g/ml LH (NIH-LH-B10; \blacksquare), 20 μ g/ml epinephrine (\square), or 10 mM NaF (\square), as indicated in *Materials and Methods*. Results are the mean \pm SD of triplicate determinations.

concentration of dibutyryl cAMP (Fig. 1C and Table 1). This effect of dibutyryl cAMP appeared to be specific for LH-stimulated cyclase, as NaF-stimulated AC activity was unchanged with all concentrations of dibutyryl cAMP (Fig. 1). In control flasks, sodium butyrate did not reduce LH-stimulated AC activity (Table 1). These data indicate that dibutyryl cAMP can promote a reduction of LH-stimulable AC activity in a time-dependent fashion.

Experiments were also conducted to evaluate the effect of cAMP on follicular AC activity. Follicles were incubated with BSA, LH, or cAMP for 1, 2, or 4-h. cAMP did not lower LH-stimulated AC activity (Table 1) or FSH-stimulated AC activity (not shown) during the 1-h incubations, while LH- and FSH-stimulated AC activities were both significantly reduced during the 2- and 4-h incubations to levels equivalent to those induced by incubation of follicles with LH (Table 1). These results

TABLE 1. Effect of *in vitro* incubation of isolated rabbit Graafian follicles with LH, dibutyryl cAMP, cAMP, and sodium butyrate on LH stimulation of AC

Incubations at 37 C			Decrease in LH-stimulated AC activity	
Additions	Time (h)	n	% of control \pm SEM ^a	P value of effect ^b
LH (10 μ g/ml)	0.5-4	16	61 \pm 4	<0.01
Dibutyryl cAMP				
0.4-1 mM	0.5-2	10	4 \pm 10	NS
4-8 mM	0.5-1	5	28 \pm 8	<0.1
4-8 mM	2-4	4	45 \pm 9	<0.05
cAMP				
20 mM	1	4	-10 \pm 9 ^c	NS
20 mM	2-4	4	43 \pm 7	<0.05
Sodium butyrate (20 mM)	2	4	-12 \pm 8 ^c	NS

^a Control activity is the activity obtained with LH in the homogenates of follicles incubated without additions. Values are the averages of paired comparisons \pm SEM of the number of comparisons shown.

^b Probability of the percent decrease being null.

^c Mean LH-stimulated activity was slightly higher than that in corresponding control flasks.

not only corroborate the findings with dibutyryl cAMP on the time dependence of the effect, but also reveal that the effect of cAMP is nonspecific, since both LH- and FSH-stimulated AC activities are reduced.

Lack of effects of inhibitors of RNA and protein synthesis on LH-induced desensitization of Graafian follicle AC

We investigated whether LH-induced desensitization of the follicular AC system could be inhibited by *in vitro* incubation of follicles with actinomycin D, cycloheximide, or puromycin. Graafian follicles were preincubated for 60-min with cycloheximide, puromycin, or actinomycin D in concentrations used effectively by other investigators (3, 8, 9), followed by a second 60-min incubation period with LH in the continued presence of these drugs. In control flasks we found no effect of preincubating Graafian follicles for 60-min with 11 μ M puromycin, 30 μ M cycloheximide, or 8 μ M actinomycin D on the responsiveness of the AC system to LH or NaF (Table 2). Further, incubation of Graafian follicles preincubated 60-min in the presence of the same inhibitors plus 10 μ g/ml LH resulted in desensitization of the AC system (Table 2). The absolute AC activity results from one of these experiments is shown in Fig. 2.

In view of the lack of effect of the above protein and RNA synthesis inhibitors on the hormonal responsiveness of the AC system and on the desensitizing action of LH, we investigated whether we indeed had inhibited amino acid incorporation into proteins and nucleotide

TABLE 2. Lack of interference of puromycin, cycloheximide, and actinomycin D with LH-induced desensitization of LH-stimulated AC activity in intact isolated Graafian follicles

Additions to the pre-desensitization incubation (at time 0) ^a	Additions to the desensitization incubation (at 60 min) ^a	n	AC activity (LH/basal; ^b fold stimulation ± SD)	P ^c
BSA (10 µg/ml)	BSA	3	11.9 ± 1.6	NS
Puromycin (11 µM)	BSA	2	8.8 ± 2.5	NS
Cycloheximide (30 µM)	BSA	2	11.3 ± 3.3	NS
Actinomycin D (8 µM)	BSA	3	17.6 ± 12.1	NS
BSA (10 µg/ml)	LH	5	1.4 ± 0.2	NS
Puromycin (11 µM)	LH	5	1.3 ± 0.2	NS
Cycloheximide (30 µM)	LH	5	1.0 ± 0.2	NS
Actinomycin D (8 µM)	LH	5	1.3 ± 0.1	NS

^a Flasks with dissected follicles in KRB-glucose were preincubated for 60 min at 37 C with the additives shown. After this, 10 µg/ml BSA or LH were added as indicated, and incubations were continued for an additional 60 min. Follicles were then removed from flasks, blotted, subjected to a further 15-min wash incubation in KRB-glucose alone, popped, homogenized, and assayed in triplicate for AC activity in the presence of BSA (basal) or LH, as described in *Materials and Methods*.

^b AC activity is expressed as the relative stimulation by LH and is calculated by dividing the absolute LH-stimulated AC activity by basal AC activity. Values are the mean ± SD of n experiments.

^c Determined by Student's *t* test. Probability that the AC activity determined in the presence of BSA or LH was not affected by preadditions of the inhibitors.

incorporation into RNA under the incubation conditions used in our experiments. Intact follicles were incubated 2-h, as described in *Materials and Methods*. Actinomycin D, puromycin D, or cycloheximide was added at time zero; ³H-labeled amino acids or [³H]uridine were added after 45-min; and LH or BSA was added after 60-min. Follicles were incubated for an additional 60-min. Follicles were then rinsed, placed in the homogenizer without prior popping, and homogenized, and the follicular RNA, DNA, and protein fractions were chemically separated in order to determine the incorporation of the radiolabeled precursors into these fractions, as described in *Materials and Methods*. We found that puromycin and cycloheximide inhibited ³H-labeled amino acid incorporation into follicular protein by 97% and 95%, respectively, and that actinomycin D inhibited [³H]uridine incorporation into follicular RNA by 100% (not shown).

Experiments were also conducted to determine whether these drugs directly modified AC activity by assaying a follicular homogenate in the presence of the drugs for basal and LH-stimulated AC activity. We found that none of the drugs significantly inhibited basal or LH-sensitive AC activity in the 10-min AC assay (not shown).

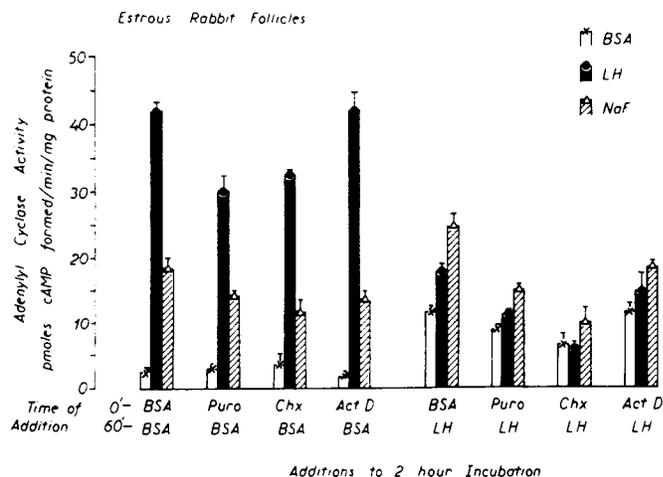


FIG. 2. Effect of *in vitro* exposure of rabbit Graafian follicles to puromycin, cycloheximide, or actinomycin D on LH-induced desensitization of the follicular AC system. Follicles from ovaries of 12 estrous rabbits (4-8 follicles/rabbit) were distributed to separate flasks. Flasks (4-5 follicles/flask, each from a separate rabbit) were incubated at 37 C for 1 h in 2.5 ml KRB-glucose containing BSA, 11 µM puromycin (Puro), 30 µM cycloheximide (Chx), or 8 µM actinomycin D (Act D), as described in *Materials and Methods*. BSA or 10 µg/ml LH (NIH-LH-S18) was then added to each flask, and incubations were continued for another hour. Follicles were then rinsed by a 15-min incubation in fresh KRB-glucose and homogenized, and follicular homogenates were assayed for AC activity in the presence of BSA (□), 10 µg/ml LH (■), or 10 mM NaF (▨), as described in *Materials and Methods*. Results are the mean ± SD of duplicate determinations. The apparent enhancement of desensitization of the LH-sensitive AC by puromycin and cycloheximide seen in the figure was not a consistent observation.

Lack of effect of colchicine, cytochalasin B, and TNP on LH-induced desensitization of Graafian follicle AC

Using an *in vitro* incubation protocol equivalent to that described in the previous sections, we investigated the effects of colchicine and cytochalasin B, drugs which disrupt the cytoskeletal system (*cf.* Ref. 10), as well as TNP, a substance which in lymphocytes and erythrocytes inhibits capping and endocytosis, presumably by directly affecting the surface area and shape of the cell membrane (11), on LH-induced desensitization of the follicular AC. Follicles were preincubated for 60 or 90-min with the drugs before LH or BSA addition. After the addition of BSA or LH, follicles were incubated for an additional 60-min, then rinsed and homogenized, and homogenates were assayed for AC activity. We found that none of these substances effected the extent of LH-induced AC desensitization induced during the second, 60-min incubation with LH (Table 3).

We also evaluated the effects of these substances directly on basal and LH-stimulated AC activity in homogenates derived from follicles which were not incubated. Neither colchicine, cytochalasin B, nor TNP elicited a significant reduction of either basal or LH-stimulated AC activity, and DMSO, the diluent for cytochalasin B,

TABLE 3. Lack of interference of colchicine, cytochalasin B, and TNP with LH-induced desensitization of LH-stimulable AC activity in intact isolated Graafian follicles

Preaddition to the desensitization incubations ^a	n	LH-stimulated AC activity (% of control \pm SEM) ^b	P ^c
None	4	62 \pm 6	<0.02
DMSO (10%)	3	62 \pm 6	<0.01
DMSO (10%) + cytochalasin B (15 μ g/ml)	3	64 \pm 6	<0.02
Colchicine (50 μ M)	4	59 \pm 8	<0.05
TNP (1-10 mM)	3	56 \pm 10	<0.05

^a Flasks with dissected follicles in KRB-glucose were incubated for 60-90 min with the additives shown. After this, 10 μ g/ml LH were added, and incubations were continued for an additional 60-min period at 37 C. Follicles were then removed, blotted, subjected to a further 15-min wash incubation to remove excess LH, popped, homogenized, and assayed for AC activities in the absence and presence of LH and NaF, as described in *Materials and Methods*. None of the treatments had an effect on fluoride-stimulated activities (not shown).

^b LH-stimulated activities are expressed as the percentage of activities determined in control follicles that had been subjected to the above incubations without addition of a desensitizing dose of LH. Values are the mean \pm SEM from three to four separate experiments.

^c Determined by Student's *t* test. Probability that the loss of activity determined in the presence of LH was not affected by preadditions.

produced a 40% increase in LH-stimulated AC activity (not shown). In other systems, colchicine and cytochalasin B are also ineffective on cell-free AC activities (12, 13).

Effect of GMP-P(NH)P on AC activity of follicular homogenates with a LH refractory AC system

The purpose of this study was to test the reversibility of desensitization by subjecting the homogenates of follicles previously desensitized to LH to a resensitizing incubation preliminary to the assay for AC activity. Graafian follicles were incubated for 2-h in the presence of BSA or a desensitizing concentration of LH. Follicles were then homogenized, and these follicle homogenates were assayed for basal and LH-stimulated AC activity in typical 10-min assays in the absence and presence of 10 μ M GMP-P(NH)P, or in a 30-min, two-stage assay. In the 10-min AC assays [conducted in the absence of GMP-P(NH)P], we found that desensitization of the AC to LH (in follicles preincubated 120-min with LH) was 85% complete (Fig. 3). The presence of GMP-P(NH)P in the 10-min assay clearly enhanced the catalytic activity of both basal and LH-stimulated AC activities of control follicles (not incubated; Fig. 3, bars 1 and 2) and follicles incubated with BSA or LH for 120-min (Fig. 3, bars 3-6); however, GMP-P(NH)P did not resensitize the desensitized follicular AC (Fig. 3, bars 5 and 6). In the 30-min, two-stage incubations, follicle homogenates were first

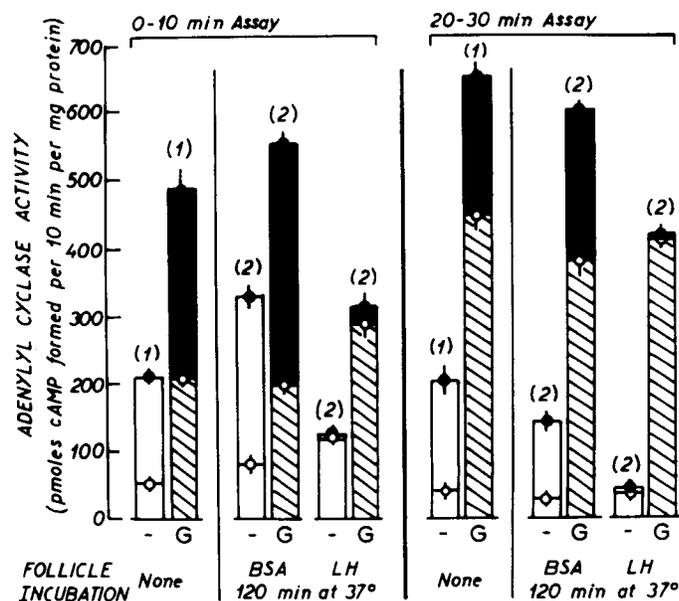


FIG. 3. Desensitization to LH in isolated rabbit Graafian follicles and failure of GMP-P(NH)P to resensitize under cell-free conditions. Follicles were dissected from rabbit ovaries and were either not incubated (None on lower abscissa) or were randomly distributed into flasks and incubated for 120 min at 37 C in the presence of 10 μ g/ml BSA (BSA on lower abscissa) or 10 μ g/ml LH (LH on lower abscissa). The incubated follicles were subjected to a 5-min wash incubation in fresh KRB-glucose without added BSA or LH. Follicles were then removed from vials, blotted and popped, and homogenized. AC activity was determined in 20- μ l homogenate aliquots a) in the absence of GMP-P(NH)P (— on abscissa) but in the presence of 10 μ g/ml BSA (\circ and \square) or 10 μ g/ml LH (\bullet and \square); or b) in the presence of 10 μ M GMP-P(NH)P (G on abscissa) and 10 μ g/ml BSA (\circ and \square) or 10 μ g/ml LH (\bullet and \square). AC activity was determined using either a 0- to 10-min assay or a 20- to 30-min assay, as indicated on the upper abscissa. The 0- to 10-min assay was the typical 10-min AC assay in which all AC reagents, including [³²P]ATP, were added at time zero, and the assay was terminated after 10 min. The 20- to 30-min assay consisted of a two-stage incubation. The first stage was carried out in the absence of [³²P]ATP but in the presence of all AC assay reagents and in the presence or absence of LH and GMP-P(NH)P, as indicated on the lower abscissa (at 1.25 times the concentration desired in the second stage of the incubation). The second stage of the 20- to 30-min assay was initiated after 20 min by the addition of [³²P]ATP and was terminated after 10 min. The numbers in parentheses represent the number of flasks containing 20 follicles (follicles in each flask were from a separate rabbit), the homogenates of which were assayed separately under 0- to 10- and 20- to 30-min assay conditions. Values represent the mean \pm SD of triplicate determinations or the mean \pm half the range of the means.

incubated for 20-min in a resensitization incubation in the presence of all AC reagents (including unlabeled ATP), except for labeled ATP and LH, and in the presence or absence of GMP-P(NH)P. The second stage was the typical 10-min AC assay and was initiated with the addition of labeled ATP and BSA or LH. We found that the 20-min resensitization incubation stage did not result in resensitization of the desensitized AC system, regardless of whether GMP-P(NH)P was present during this

time (Fig. 3, last two bars). Since the 20-min resensitization incubation stage also contained ATP (3.75 mM), it is clear that ATP is also unable to promote resensitization (Fig. 3, bars 11 and 12). Similar results have been reported with rat follicles by Lamprecht *et al.* (3) and with luteinized rat ovaries by Conti *et al.* (14).

Discussion

Recent studies have shown that gonadotropin-induced AC desensitization in follicle shells derived from Graafian rabbit follicles occurs in a biphasic manner (15). The initial phase is specific for LH and occurs during the first hour after hCG injection or coitus. In the secondary phase of desensitization, the follicle shell AC also exhibits a decreased response to highly purified FSH. In the present manuscript, we investigated possible mechanisms of LH-induced desensitization of the Graafian follicle AC during both phases of desensitization.

We show that the initial 60-min phase of LH-induced desensitization of the LH-sensitive AC system in rabbit follicles does not require RNA or protein synthetic events, based upon studies in which we effectively blocked RNA and protein synthesis with actinomycin D, puromycin, and cycloheximide. This is not a surprising result in view of the rapid rate at which AC desensitization occurs in rabbit follicles. Yet, LH-induced desensitization in rat follicles, a seemingly similar system, requires an actinomycin D-sensitive step (3), while FSH-induced desensitization in cultured granulosa cells does not exhibit this requirement (16). Our results in rabbit follicles and those of Nimrod and Lamprecht (16) in rat granulosa cells are not necessarily incompatible with the rat follicular studies of Lamprecht *et al.* (3). AC desensitization in rat follicles, whether obtained *in vivo* (2) or *in vitro* (17), is a much slower event than in rabbit follicle shells (1, 15) or rat granulosa cells (16), and it is not unlikely that AC desensitization in these seemingly similar systems is mediated by different mechanisms. Indeed, there is a controversy in a variety of systems in the literature regarding the requirement of RNA and/or protein synthetic processes in AC desensitization (*cf.* Ref. 18). Generally, in those systems in which the effect of RNA and/or protein synthesis inhibitors on AC desensitization has been evaluated, when AC desensitization is a rapid event (>50% refractoriness in 2-h), RNA and/or protein synthesis inhibitors are often without effect (8, 16, 19–22). Similarly, recent studies in enucleated myeloid leukemic cells indicate that AC desensitization is an extranuclear process (23). There are, however, examples of systems in which AC desensitization occurs rapidly yet is blocked by RNA and/or protein synthetic drugs (3, 24, 25). Conversely, when AC desensitization is a slower event (50% refractoriness requiring 12-h or more), RNA

and/or protein synthesis inhibitors block AC desensitization (3, 26). It would seem that the presence of a refractory AC system is only an indication of a final event, an event which may be mediated by different processes in different systems.

We also show that the initial hormone-specific phase of LH-induced desensitization of the rabbit follicular AC system to LH does not require an intact cytochalasin B-sensitive microtubular or colchicine-sensitive microfilamentous cytoskeletal structure, nor does AC desensitization in rabbit Graafian follicles require surface membrane events which in lymphocytes and erythrocytes are blocked by TNP. Also, in S49 lymphoma cells, colchicine does not prevent AC desensitization to isoproterenol (13), while in myeloid leukemic cells, colchicine, but not cytochalasin B inhibits the desensitization response to prostaglandin E₁ and isoproterenol (23). Since both cytochalasin B and colchicine were used in our studies in a concentration range which disrupts microfilaments and microtubules in a variety of other systems (10, 27–30), our results suggest that the initial, hormone-specific phase of desensitization of the follicular AC system in Graafian follicles is not the result of an endocytotic process in which one or more of the AC system components are internalized.

In some systems, a more prolonged incubation of intact cells with colchicine often enhances cAMP production (12, 13, 23, 31), and in others, prolonged incubation with cytochalasin B inhibits LH stimulation of the AC system (10). We observed no significant enhancement or inhibition of cAMP production by rabbit Graafian follicles during 2- or 2.5-h incubations with these drugs.

A 2-h exposure of rabbit Graafian follicles to exogenous cAMP or dibutyryl cAMP, in concentrations which promote luteinization of and steroidogenesis by estrous rat and rabbit follicles (23–34), caused significant reduction ($P < 0.05$) in the LH-stimulated AC activity of follicle homogenates. Nearly total desensitization of the LH-sensitive AC was observed upon exposure of Graafian follicles to dibutyryl cAMP for 4 h; however, 30-min and 1-h exposures were without effect on LH-stimulated AC activity. Likewise, in other systems, exposure to cAMP or its dibutyryl derivative for 20–30 min was without effect (21, 35), while more prolonged exposure promoted AC refractoriness, often of a nonspecific nature (20, 25, 36, 37). However, prolonged exposure to dibutyryl cAMP does not promote AC desensitization in all systems (38).

These *in vitro* studies with Graafian follicles reveal that the initial 60- to 90-min, hormone-specific phase (13) of LH-induced desensitization of the AC system does not require RNA or protein synthetic events, nor does it depend upon components of the cytoskeletal system. Our recent studies in a porcine follicular membrane system, in which partial AC refractoriness to LH occurs within

20-min, indicate that the activity of the AC system may be regulated by the level of phosphorylation of membrane-associated components of the AC system (39). The ability of cAMP and its dibutyryl analog to promote a decline in LH-stimulated AC activity after 2-h *in vitro* suggests that a second component of the AC desensitization mechanism may be cAMP feedback upon the AC system.

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