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Effect of a Phosphodiesterase Inhibitor, 3-Isobutyl 1-methylxanthine, upon the Stimulatory Effect of Human Follicle-Stimulating Hormone and Human Luteinizing Hormone upon Cyclic Adenosine 3′:5′-Monophosphate Accumulation by Porcine Granulosa Cells[1] (40321)

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A mechanism of polypeptide hormone action on target cells is to stimulate formation of cAMP which subsequently acts as an intracellular mediator of hormone action. Intracellular cAMP levels are the result of given rate of synthesis combined with a given rate of degradation or extracellular release. The cyclic nucleotide is believed to be hydrolyzed to 5′-AMP by one or more cyclic nucleotide phosphodiesterases (1). Methylxanthines have been shown to exert inhibitory effects on the action of phosphodiesterase (2, 3). We have shown previously that LH and FSH can stimulate cAMP accumulation by porcine granulosa cells (GC) and that the amount of cAMP accumulated in response to the two gonadotropins differs according to the stage of maturation of the follicle (4). In addition, observations from previous studies (4) suggest that the phenomenon of cAMP accumulation by porcine GC in response to the stimulatory effects of the gonadotropins occurs over time. For GC from small and medium follicles the intracellular cAMP accumulated in response to FSH was not observed to decline significantly in incubations of 30 min or less. The decline occurred between 30- and 60-min periods of incubation and it was during this time interval that the increase in cAMP accumulation in the incubation medium was observed to occur. For GC from large follicles the intracellular cAMP accumulated in response to LH was not observed to decline with 30- nor with 60-min incubations; however, a significant increase in the cAMP accumulation in the incubation medium occurred between 30- and 60-min periods of incubation. The present studies were designed to investigate the influence that phosphodiesterase may exert on the cAMP accumulation phenomenon previously observed in porcine GC in response to the stimulatory effects of FSH and LH. In the present studies the phosphodiesterase influence was examined indirectly using a potent phosphodiesterase inhibitor.

The effects of phosphodiesterase inhibition upon cAMP accumulation by porcine GC previously have not been adequately examined. The influence of methylxanthine upon the stimulatory effects of purified hFSH and hLH on porcine GC intracellular cAMP accumulation and upon cAMP accumulation in the incubation medium was investigated. These studies enabled the determination of the relative approximate contribution of synthesis, degradation, and extracellular release to cAMP levels occurring in porcine GC during various stages of follicular maturation in response to hFSH and hLH.

Materials and methods. Granulosa cell harvest. Porcine ovaries were obtained from a local meat packing plant within 15 to 20 min of sacrifice of the animals. Granulosa cells were harvested from small (1–2 mm), medium (3–5 mm), and large (6–12 mm) follicles according to the method of Channing and Ledwitz-Rigby (5). Using dye exclusion as an indication of cell viability, the cells were counted in a hemocytometer in 0.06% trypan blue.

Hormones and chemicals. Highly purified hLH, LER-1705, having a potency of 3800 IU/mg and an FSH activity of 3 IU/mg, and hFSH, LER-1577[c], having an FSH potency of 880 IU/mg were used. These two hormone preparations were provided by Dr. L. E. Reichert, Jr. The FSH preparation as sup-

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plied by Dr. Reichert had been pretreated with chymotrypsin to inactivate the contaminating LH selectively (6). The residual LH activity is reported to be 5.7 IU/mg using the ovarian ascorbic acid depletion assay (7). According to Amir *et al.* (8), controlled chymotrypsin digestion does not destroy FSH activity as determined by the Steelman–Pohley bioassay (9).

The cells were incubated in the absence or presence of the hormones in Eagle's medium containing Earle's salts (pH 7.4; Grand Island Biological Co., Grand Island, N.Y.), 25 m*M* HEPES buffer (Calbiochem), 2.2 g/liter $NaHCO_3$ (Grand Island Biological Co.), and 1% bovine serum albumin (BSA) fraction V (Sigma Chemical Company). This was designated Eagle's medium plus 1% BSA. 3-Isobutyl 1-methylxanthine (MIX) was purchased from the Aldrich Chemical Company (Milwaukee) and was diluted in Eagle's medium plus 1% BSA. The final concentration employed for incubations with the cells and gonadotropins was 0.2 m*M*. Both [^{3}H]cAMP (sp act 24 Ci/mmol) and nonlabeled cAMP were purchased from Schwartz Bio-Research, Inc.

Granulosa cell incubations and experimental procedures. Granulosa cells from small and medium follicles were suspended in Eagle's medium plus 1% BSA and dispensed in aliquots of 2×10^7 cells. Cells from large follicles were dispensed in aliquots of 5×10^6 cells. Incubations were carried out in Packard glass scintillation vials containing the appropriate hormone. When 3-isobutyl 1-methylxanthine (MIX) was used it was added to the vials containing the appropriate hormone and to vials containing no hormone prior to addition of the cells. The final incubation volume per vial was 1.0 ml. Three to five replicate aliquots of cells were used for each variable in each experiment. Incubations were carried out for 30 and 60 min under conditions previously described (5). The reaction was arrested by placing the vials immediately in ice. The cells were separated from the incubation medium by centrifugation; the incubation medium was decanted and frozen for later assay of cAMP content. The remaining cell pellets were subjected to hot sodium acetate extraction and following centrifugation the clear supernatant was decanted and frozen for later assay of intracellular cAMP.

Cyclic AMP assay. Cyclic AMP accumulation was determined by a competitive protein binding assay (10) with modifications (5, 11). Using 1.25 pmol of cAMP as a standard after every 10 unknown samples, the intra-assay coefficient of variation was less than 16% and for 30 randomly selected assays the between assay coefficient of variation was less than 15%.

Results. Effect of MIX upon intracellular cAMP accumulation. The presence of 0.2 m*M* MIX in the incubation medium did not significantly alter the control levels of intracellular cAMP in GC harvested from small, medium, and large follicles following 30- or 60-min incubation periods (Table I). Addition of 1.0 and 10 μg of hFSH resulted in an increase in intracellular cAMP accumulation in GC from small, medium, and large follicles (Table I). In the case of cells from small follicles, addition of 10 μg of hFSH led to a greater than 13-fold increase ($p < 0.001$) in intracellular cAMP levels following 30-min incubations and a greater than 22-fold increase ($p < 0.001$) following a 60-min incubation period (Tables I and II). In contrast, addition of 10 μg of hFSH to cells from large follicles led to less than a 3-fold increase above control levels after either 30- or 60-min incubation periods ($p < 0.001$ and $p < 0.01$, respectively). A small nonsignificant ($p > 0.05$) potentiating effect of 0.2 m*M* MIX upon the stimulatory effect of 1.0 and 10 μg of hFSH upon intracellular cAMP accumulation was observed (Table I).

Addition of hLH stimulated intracellular cAMP accumulation in GC (Table I). The stimulation was greater in the case of GC harvested from large compared to medium and small follicles. Addition of 0.2 m*M* MIX exerted a small nonsignificant ($p > 0.05$) potentiating effect upon the LH stimulation of intracellular cAMP levels in cells from all three types of follicles (Table I). If GC were incubated for 60 rather than 30 min addition of 0.2 m*M* MIX still had no significant effect upon hFSH and hLH stimulation of intracellular cAMP levels (Table II).

Effect of MIX upon cAMP released into the incubation medium. The presence of 0.2 m*M* MIX in the incubation medium did not sig-

TABLE I. COMPARISON OF EFFECT OF 0.2 m*M* 3-ISOBUTYL 1-METHYLXANTHINE UPON hFSH AND hLH STIMULATION OF INTRACELLULAR cAMP ACCUMULATION IN PORCINE GC DURING 30-MIN INCUBATIONS.[a]

Source of GC and treatment	Intracellular cAMP (pmol/5 × 10^7 cells)	
	3-Isobutyl 1-methylxanthine	
	Absent	Present
Small follicle		
Control	8.1 ± 0.7	8.9 ± 0.9
0.1 μg hFSH	10.4 ± 1.3	14.2 ± 2.8
1.0 μg hFSH	62.7 ± 3.7	71.0 ± 5.6
10.0 μg hFSH	91.5 ± 9.4	99.3 ± 9.3
0.01 μg hLH	8.6 ± 2.2	8.7 ± 1.3
0.1 μg hLH	9.5 ± 1.9	12.7 ± 1.9
1.0 μg hLH	12.7 ± 2.1	15.4 ± 2.1
Medium Follicle		
Control	8.9 ± 1.6	8.4 ± 1.2
0.1 μg hFSH	8.0 ± 0.3	9.9 ± 0.5*
1.0 μg hFSH	38.3 ± 6.1	37.6 ± 4.5
10.0 μg hFSH	51.6 ± 4.2	40.4 ± 6.9
0.01 μg hLH	13.2 ± 3.6	11.3 ± 1.0
0.1 μg hLH	23.5 ± 3.4	30.6 ± 3.2
1.0 μg hLH	30.8 ± 1.4	36.9 ± 3.5
Large follicle		
Control	92.9 ± 6.2	109.6 ± 8.2
0.1 μg hFSH	85.9 ± 14.8	95.0 ± 17.7
1.0 μg hFSH	184.5 ± 10.3	191.0 ± 21.8
10.0 μg hFSH	204.3 ± 9.9	231.8 ± 20.5
0.01 μg hLH	134.3 ± 10.0	149.4 ± 31.0
0.1 μg hLH	236.5 ± 12.0	272.1 ± 13.9
1.0 μg hLH	278.1 ± 12.4	314.5 ± 13.4

[a] Data are expressed as the means ± SE of four observations. Granulosa cells harvested from small, medium, and large porcine follicles were incubated for 30 min with hFSH or hLH in the absence or presence of 0.2 m*M* MIX and the intracellular cAMP levels were determined. Student's *t* test was used to compare results (MIX present vs MIX absent). The differences were not statistically significant ($p > 0.05$) unless indicated.

* $p < 0.05$.

nificantly alter control levels of cAMP released into the incubation medium by GC from any size follicle during 30- or 60-min incubation periods (Tables III and IV). Addition of 10 μg of hFSH to GC from small follicles led to a 16- and 45-fold increase in incubation medium cAMP levels following 30- and 60-min incubation periods, respectively (Tables III and IV). In the case of GC from small and medium follicles, addition of 0.2 m*M* MIX in the presence of 1.0 (data not shown) and 10 μg of hFSH led to a significant increase in incubation medium cAMP content (Tables III and IV). In contrast, the presence of MIX did not significantly potentiate the effect of hFSH upon cAMP accumulation in the incubation medium by GC from large follicles (Tables III and IV).

The presence of MIX brought about a significant potentiation of the stimulatory effect of 1.0 μg of hLH upon cAMP released into the incubation medium by GC from small and medium follicles following 30- and 60-min incubations (Tables III and IV). In the case of GC from large follicles the potentiating effect of MIX upon hLH stimulation of cAMP accumulation in the incubation medium was not significant ($p > 0.05$) during 30- or 60-min incubations (Tables III and IV).

After a 60-min incubation period with either 10 μg of hFSH or 1.0 μg of hLH the incubation medium cAMP levels were consistently greater than the intracellular levels in the case of cells from all three follicle types (Tables II and IV).

TABLE II. COMPARISON OF EFFECT OF 0.2 m*M* 3-ISOBUTYL 1-METHYLXANTHINE UPON hFSH AND hLH STIMULATION OF INTRACELLULAR cAMP ACCUMULATION IN PORCINE GC DURING 60-MIN INCUBATIONS.[a]

Source of GC and treatment	Intracellular cAMP (pmol/5 × 10^7 cells) 3-Isobutyl 1-methylxanthine Absent	Present
Small follicle		
Control	3.1 ±0.2	4.3 ±0.7
10.0 μg hFSH	71.0 ±4.8	75.8 ±3.7
1.0 μg hLH	10.8 ±0.8	13.6 ±3.2
Medium follicle		
Control	3.8 ±0.3	4.1 ±0.2
10.0 μg hFSH	22.0 ±3.64	28.1 ±3.7
1.0 μg hLH	11.1 ±2.0	13.0 ±3.5
Large follicle		
Control	86.8 ±6.6	61.5 ±6.3
10.0 μg hFSH	224.1 ±30.6	264.6 ±34.6
1.0 μg hLH	369.1 ±25.2	385.5 ±44.6

[a] Data are expressed as the means ± SE of four observations. Granulosa cells harvested from small, medium, and large porcine follicles were incubated for 60 min with hFSH or with hLH in the absence or presence of 0.2 m*M* MIX and the intracellular cAMP levels were determined. Student's *t* test was used to compare results (MIX present vs MIX absent). The differences were not statistically significant ($p > 0.05$).

Discussion. The lack of a significant potentiating effect of MIX on intracellular cAMP accumulation by porcine GC in response to 30- or 60-min periods of incubation with either hFSH or hLH could indicate that enzymatic hydrolysis of cAMP by a phosphodiesterase(s) is not a major mechanism responsible for controlling the intracellular levels of the cyclic nucleotide. Alternatively, it is possible that this methylxanthine does not readily permeate the GC plasma membrane and successfully inhibit phosphodiesterase or that the concentration employed was not sufficient to inhibit GC intracellular phosphodiesterase(s). It is evident from the findings of other investigators that concentrations of MIX ranging from 0.01 to 1.0 m*M* have potentiating effects on cAMP accumulation. Methylxanthine has been observed to potentiate the effect of ACTH upon cAMP levels in rat adrenal homogenates and quarters (13) and in isolated fat cells (14). Mendelson *et al.* (12) reported that the sensitivity of isolated rat testis interstitial cells to hCG stimulation was significantly enhanced with the presence of 0.1 m*M* MIX and in the absence of MIX, cAMP accumulation in response to hCG was reduced in magnitude by about 60%. These investigators used the sonicated incubation mixture for assay of cAMP; thus their reported findings reflect inclusion of both the intracellular and incubation medium cAMP content and the site of the potentiating effect remains obscure. Channing (15) observed

TABLE III. COMPARISON OF EFFECT OF 0.2 m*M* 3-ISOBUTYL 1-METHYLXANTHINE UPON hFSH AND hLH STIMULATION OF cAMP ACCUMULATION IN THE INCUBATION MEDIUM BY PORCINE GC DURING 30-MIN INCUBATIONS.[a]

Source of GC and treatment	Incubation medium cAMP (pmol/5 × 10^7 cells) 3-Isobutyl 1-methylxanthine Absent	Present
Small follicle		
Control	7.0 ±0.7	6.9 ±0.8
10.0 μg hFSH	115.3 ±3.2	143.2 ±3.5***
1.0 μg hLH	6.2 ±0.7	13.4 ±1.4***
Medium follicle		
Control	6.5 ±0.6	7.1 ±0.8
10.0 μg hFSH	25.1 ±2.9	49.3 ±3.4***
1.0 μg hLH	10.8 ±1.6	19.8 ±1.4***
Large follicle		
Control	26.3 ±4.9	27.6 ±4.6
10.0 μg hFSH	110.2 ±19.5	158.0 ±26.4
1.0 μg hLH	210.9 ±62.8	269.1 ±66.3

[a] Data are expressed as the means ± SE of eight observations. Granulosa cells harvested from small, medium, and large porcine follicles were incubated for 30 min with hFSH or with hLH in the absence or presence of 0.2 m*M* MIX and the incubation medium cAMP levels were determined. Student's *t* test was used to compare results (MIX present vs MIX absent).

*** $p < 0.001$.

TABLE IV. COMPARISON OF EFFECT OF 0.2 m*M* 3-ISOBUTYL 1-METHYLXANTHINE UPON hFSH AND hLH STIMULATION OF cAMP ACCUMULATION IN THE INCUBATION MEDIUM BY PORCINE GC DURING 60-MIN INCUBATIONS.[a]

Source of GC and treatment	Incubation medium cAMP (pmol/5 × 10^7 cells)	
	3-Isobutyl 1-methylxanthine Absent	Present
Small follicle		
Control	4.4	6.6
	±0.4	±1.8
10.0 μg hFSH	198.6	297.7
	±12.2	±19.5**
1.0 μg hLH	28.2	68.3
	±2.3	±3.4***
Medium follicle		
Control	11.7	13.0
	±3.8	±4.8
10.0 μg hFSH	101.1	146.0
	±16.6	±5.4*
1.0 μg hLH	38.9	77.9
	±5.4	±3.6***
Large follicle		
Control	77.6	72.6
	±15.5	±12.8
10.0 μg hFSH	591.3	639.4
	±21.1	±38.6
1.0 μg hLH	830.9	810.8
	±52.2	±55.3

[a] Data are expressed as the means ± SE of four observations. Granulosa cells harvested from small, medium, and large porcine follicles were incubated for 60 min with hFSH or with hLH in the absence or presence of 0.2 m*M* MIX and the incubation medium cAMP levels were determined. Student's *t* test was used to compare results (MIX present vs MIX absent).

* $p < 0.05$.

** $p < 0.01$.

*** $p < 0.001$.

that in 20-min incubations of porcine GC from medium-sized follicles, addition of 3.0 m*M* aminophylline to incubation medium containing either FSH or LH significantly increased the concentration of intracellular cAMP when compared to the effect of FSH or LH alone. The difference in these findings and the results observed in the present studies could be due to differences in the effect of the two inhibitors on GC phosphodiesterase activity; it is possible that aminophylline has a synergistic effect with the gonadotropins in stimulating cAMP production. In another series of experiments, addition of theophylline alone without gonadotropins to incubations of isolated prepubertal rat ovaries resulted in a stimulation of cAMP accumulation significantly above control levels in both the tissue and in the incubation medium (16). The effects of theophylline could have been due to the indirectly mediated inhibitory influence upon protein synthesis or due to a direct inhibition of phosphodiesterase (17).

If cAMP is protected from the hydrolytic action of phosphodiesterase by subcellular compartmentalization in GC, inhibition of the degradative enzymatic activity by methylxanthine would not be significantly apparent. Cheung (18) has shown that cAMP bound to the protein kinase regulatory subunit is not susceptible to phosphodiesterase activity and only is degraded when dissociated from the protein. It was concluded that the rate of hydrolysis of cAMP is governed by its rate of dissociation from the protein kinase regulatory subunit. In the present studies it is possible that the lack of a significant potentiating effect of methylxanthine upon gonadotropin stimulation of intracellular cAMP accumulation could have resulted from cAMP being bound to the protein kinase regulatory subunit during the time intervals examined. Means *et al.* (19, 20) observed that when testis were incubated for 1 hr with FSH, the protein kinase remained maximally active following an additional 2 hr of incubation without the gonadotropin present. Similar compartmentalization of intracellular cAMP may occur in porcine GC and explain the lack of a significant potentiating response of the phosphodiesterase inhibitor.

The finding that methylxanthine has a significant potentiating effect upon cAMP content in the incubation medium in response to either hFSH or hLH stimulation could be due to the presence of plasma membrane fragments in the incubation medium which makes the phosphodiesterase more accessible to the inhibitory action of MIX. Alternatively, it is possible that an extracellular phosphodiesterase may exist and have a role in the degradation of cAMP released from the GC. It is apparent from these and previous studies (4) that significant concentrations of cAMP are released extracellularly by porcine GC in response to the stimulatory action of the gonadotropins. Enzymatic degradation of extracellular cAMP has been reported for

incubations of prepubertal rat ovaries using the disappearance of labeled cAMP as well as production of labeled products of cAMP degradation, indicating that cAMP released into the incubation medium was undergoing extracellular degradation by a phosphodiesterase (21).

Factors influencing the intracellular location, the extracellular release, plasma membrane permeability, and metabolism of cAMP in porcine GC require more definitive studies before the questions posed can be resolved.

The possibility does exist that the MIX could have side effects other than inhibition of phosphodiesterase.

Summary. In order to examine a possible role of phosphodiesterase in mediation of the action of LH and FSH upon granulosa cell cAMP levels, porcine (GC) from small (1–2 mm), medium (3–5 mm), and large (6–12 mm) follicles were incubated with human FSH (hFSH) and LH (hLH) for 30 and 60 min in the absence or presence of 3-isobutyl 1-methylxanthine (MIX), a potent phosphodiesterase inhibitor. Subsequently, the intracellular and incubation medium cAMP contents were determined by a protein binding assay. During a 30-min incubation, 10 μg of hFSH alone brought about an 11-fold, 5-fold, and 2-fold increase in intracellular cAMP accumulation and a 16-fold, approximately 4-fold, and 4-fold increase in incubation medium cAMP levels in GC from small, medium, and large follicles, respectively. Addition of 0.2 m*M* MIX exerted a nonsignificant ($P > 0.05$) potentiating effect upon hFSH stimulation of intracellular cAMP accumulation in cells obtained from the three types of follicles. In the case of cells obtained from small and medium follicles, addition of 0.2 m*M* MIX in the presence of 10 μg/ml hFSH or 1.0 μg/ml hLH led to 41 to 69% potentiation ($p < 0.001$) of the effect of the FSH and LH upon cAMP accumulation in the incubation medium. This was evident after a 30- and 60-min incubation period. In the case of cells obtained from large follicles, addition of 0.2 m*M* MIX had a nonsignificant potentiating effect ($p > 0.05$) on either hFSH or hLH stimulation of cAMP accumulation in the incubation medium.

It may be concluded that probably there are low levels of intracellular phosphodiesterase in porcine granulosa cells and that gonadotropins act to stimulate the generation of cAMP rather than alter the rate of destruction of cAMP. The findings support the existence of an extracellular phosphodiesterase which may act to regulate or modulate the extracellular levels of cyclic AMP.

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