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Cyclic Nucleotides, Adenylate Cyclase, and Cyclic AMP Phosphodiesterase in Mammary Glands from Pregnant and Lactating Mice[1] (39299)

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Previous studies have shown that the metabolism of cyclic nucleotides in mammary glands was markedly altered during pregnancy and lactation. In mammary glands of mice, guanylate cyclase and cyclic GMP phosphodiesterase activities increased about twofold during pregnancy and following parturition (1). In mammary glands of rats, Sapag-Hagar and Greenbaum (2, 3) have reported that cyclic AMP concentrations increased about fourfold during pregnancy, but following parturition, the cyclic AMP concentration falls precipitously. Accordingly, they reported that adenylate cyclase activities paralleled the rise and fall of cyclic AMP levels during pregnancy and lactation. Cyclic AMP phosphodiesterase activities, however, increased during pregnancy but did not decrease during lactation. In contrast to cyclic AMP, levels of cyclic GMP declined during the gestation period, but immediately following parturition and during the lactation period, levels of cyclic GMP were significiantly elevated.

The present studies were carried out to measure levels of cyclic nucleotides and activities of adenylate cyclase and cyclic AMP phosphodiesterase in mammary glands of mice during pregnancy and lactation. The results were then correlated with those of Sapag-Hagar and Greenbaum (2, 3) and with previous results from this laboratory (1).

Materials and methods. Virgin and timed-pregnant, Swiss-Webster mice were purchased from Spartan Research Animals, Inc., Haslett, Mich. Mammary glands were excised from these animals within a 1 to 2 min period. Cyclic nucleotide concentrations or enzyme activities were then measured by the following methods.

Radioimmunoassay kits purchased from the Schwartz/Mann Company were used to measure tissue levels of cyclic AMP (4) and cyclic GMP (5). Following excision, the tissues were frozen in liquid nitrogen and stored at −80° until assayed for cyclic nucleotides; tissues were stored for up to 2 months prior to making measurements. The tissues were prepared for the radioimmunoassays by the following methods. The frozen tissues were weighed (30–100 mg) and homogenized in 1 ml 6% TCA. Then, after centrifugation at 2000*g* for 10 min, the supernatants were extracted three times with 3 ml of water-saturated ether. After removal of the ether, the extracts were then appropriately diluted for the radioimmunoassays.

Adenylate cyclase activity was measured by the rate of conversion of α-labeled [^{32}P]ATP to [^{32}P]cyclic AMP using the methods of Krishna *et al.* (6). Following excision, the mammary tissues were weighed and homogenized 1:10 (wt/vol) in medium containing 1 m*M* $MgCl_2$ and 0.04 *M* Tris-HCl (pH 7.5 at 30°). 0.1 ml of the homogenate was then added to reaction tubes, on ice, to which 0.05 ml of incubation medium had previously been added. The final concentrations of substances in the 0.15 ml reaction mixtures were as follows: 5 m*M* $MgCl_2$, 2 m*M* cyclic AMP (Sigma), 0.15–0.20 m*M* ATP (Sigma), 3–7 μCi/ml of [α-^{32}P]ATP (New England Nuclear), 6.7 mg/ml creatine phosphate (Sigma), 0.33 mg/ml creatine kinase (Sigma), 0.33 mg/ml bovine serum albumin (Sigma), and 40 m*M* Tris-HCl (pH 7.5 at 30°). Each homogenate was assayed in duplicate and reaction blanks were obtained by boiling portions of homogenates prior to addition to the reaction tubes. The reactions were carried out by placing the reaction tubes in a 30° waterbath for 10 min with shaking. Reactions were terminated by placing the reaction tubes in a boiling water bath for 3 min. One-

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tenth milliliter of 1 m*M* [^{3}H]cyclic AMP (New England Nuclear) containing about 10,000 cpm, was then added to each reaction tube. The [^{3}H]cyclic AMP was added for the subsequent correction for losses of [^{32}P]cyclic AMP during the procedure used to isolate the cyclic AMP. The cyclic AMP was isolated by ion exchange chromatography and a $Ba(OH)_2$-$ZnSO_4$ precipitation (6). The results are expressed as picomoles of cyclic AMP formed per minute per milligram wet tissue weight. In preliminary experiments, the use of this adenylate cyclase assay for mammary gland tissue was validated by demonstrating that (i) the formation of [^{32}P]cyclic AMP was linear with time for up to 10 min and (ii) the rat of [^{32}P]cyclic AMP formation was proportional to the amount of homogenate added to the reaction tubes.

Cyclic AMP phosphodiesterase activities were measured at two substrate concentrations, 10 or 100 μM cyclic AMP, by a combination of the methods of Beavo *et al.* (7) and Murad *et al.* (8). Mammary glands were initially weighed and homogenized 1:10 (wt/vol) at 0° in medium containing 0.25 *M* sucrose and 40 m*M* Tris–HCl (pH 7.5 at 30°). This homogenate was further diluted 1:5 when enzyme activity was measured at the low substrate concentration. The reactions mixtures were prepared in tubes, on ice, by adding 0.05 ml of 10 m*M* $MgCl_2$, 0.1 ml of [^{3}H]cyclic AMP (0.5 μCi/ml) at the appropriate concentration, and 0.1 ml of the homogenate. Enzyme reactions were carried out by placing the reaction tubes in a 30° water bath with shaking for 3 min (low substrate concentration) or 6 min (high substrate concentration). The reactions were terminated by placing the reaction tubes in boiling water bath for 3 min, 0.05 ml of 1.5 mM cyclic AMP–5 mM 5′AMP (Sigma) and 0.05 ml crotalus atrox snake venom (Sigma, 2.0 mg/ml in 0.5 *M* Tris–HCl, (pH 8.0 at 37°) were then added to the tubes and an additional incubation was carried out at 37° for 20 min. The [^{3}H]adenosine present in the reaction mixtures was then isolated by elution with 26 ml of 40 m*M* Tris–HCl (pH 7.5 at 25°) through Dowex ion-exchange columns (7). Radioactivity in the eluates was determined by liquid scintillation spectrometry. Results are expressed as picomoles of cyclic AMP converted to 5′AMP per minute per milligram wet tissue weight. This assay was validated for mammary gland tissues by the criteria described above for adenylate cyclase.

Results. The levels of cyclic AMP and cyclic GMP in mammary glands of virgin, pregnant, and lactating mice are shown in Table I. Levels of cyclic AMP appear to rise progressively during pregnancy and then fall precipitously following parturition. In contrast, cyclic GMP concentrations decreased on day 18 of pregnancy relative to the concentration on Days 14–16 of pregnancy. Following parturition, however, there was a twofold increase in the tissue content of cyclic GMP. These results are similar to the observations of Sapag-Hagar and Greenbaum (2, 3) in studies on the mammary glands from rats.

Tables II and III show, respectively, the enzyme levels of adenylate cyclase and cyclic AMP phosphodiesterase in mammary glands of virgin, pregnant, and lactating mice. There was a significant elevation of adenylate cyclase activity in the 1-day lactating mice vs the virgins, but other possible differences were not detectable because of the high degree of variability among animals (Table II). This variability was not due to our assay system since values from replicate assays were essentially the same. In any case, we did not observe the several fold increase of adenylate cyclase activity on the day immediately prior to parturition which was observed in rats by Sapag-Hagar and Greenbaum (2, 3). Day 20 was the last day of gestation in their rats, whereas Day 18 was the last day of gestation in the mice which we used. Cyclic AMP phosphodiesterase activities were elevated in pregnant and lactating mice when either the low or high substrate concentrations of cyclic AMP were tested (Table III). These results are essentially the same as those reported for rat mammary glands (2, 3).

Discussion. It has now been shown in the mammary glands of both rats and mice that the ratio of cyclic AMP to cyclic GMP rises during the gestation period of these animals, but falls markedly following parturition. It is indeed possible that elevated levels of cyclic

TABLE I. CYCLIC AMP AND CYCLIC GMP IN MAMMARY GLANDS OF VIRGIN, PREGNANT, OR LACTATING MICE.[a]

Physiological state of mice	Cyclic nucleotide concentration (pmole/mg wet tissue wt)	
	Cyclic AMP	Cyclic GMP
Virgin	0.94 ± 0.12 (12)[b]	0.066 ± 0.024 (11)
14–16 days pregnant	1.65 ± 0.21 (5)	0.090 ± 0.018 (12)
18 days pregnant	3.46 ± 0.19 (5)	0.048 ± 0.012 (5)
1–3 days lactating	1.82 ± 0.14 (12)	0.186 ± 0.030 (16)

[a] Cyclic AMP and cyclic GMP were measured by methods described in the text.
[b] Numbers in the table are means ± standard error of the number of animals indicated in the parentheses.

TABLE II. ADENYLATE CYCLASE ACTIVITY IN MAMMARY GLANDS FROM VIRGIN, PREGNANT, AND LACTATING MICE.[a]

Physiological state of mice	Adenylate cyclase activity (pmole/min/mg wet tissue wt)
Virgin	0.354 ± 0.048 (10)[b]
11–12 days pregnant	0.511 ± 0.076 (6)
15–16 days pregnant	0.551 ± 0.084 (10)
18 days pregnant	0.541 ± 0.072 (14)
1 day lactating	0.800 ± 0.117 (12)
2–3 days lactating	0.583 ± 0.089 (10)
4–5 days lactating	0.585 ± 0.078 (3)

[a] Tissues were excised and adenylate cyclase activity was measured using methods described in the text.
[b] Numbers in the table are the means ± standard errors of the number of animals shown in parentheses.

TABLE III. CYCLIC AMP PHOSPHODIESTERASE ACTIVITY IN MAMMARY GLANDS FROM VIRGIN, PREGNANT, AND LACTATING MICE.[a]

Physiological State of Mice	Cyclic AMP phosphodiesterase activity (pmole/min/mg wet tissue wt)	
	10 μM cyclic AMP	100 μM cyclic AMP
Virgin (7)	23.9 ± 1.1[b]	95.2 ± 3.2
12–14 days pregnant (8)	36.5 ± 2.0	143 ± 6
18 days pregnant (11)	32.7 ± 4.5	137 ± 13
1 day lactating (4)	35.8 ± 2.0	181 ± 9.3
3 days lactating (7)	36.9 ± 6.3	154 ± 6.9

[a] Tissues were excised and cyclic AMP phosphodiesterase was measured by methods described in the text.
[b] Numbers in the table are the means ± standard error of the number of animals indicated in the parentheses.

AMP may prevent the onset of lactation during pregnancy. Several laboratories have shown that incubation of mammary glands with agents which elevate intracellular levels of cyclic AMP results in a reduced rate of RNA synthesis (9, 10), casein synthesis (11), lactose synthesis (12), fatty acid synthesis (10), DNA synthesis (10), and the activities of several enzymes (10). Following parturition, however, the onset of lactation may be triggered by the elevated levels of cyclic GMP and the reduced levels of cyclic AMP. Supporting this contention is the observation that cyclic GMP mimics the action of prolactin on RNA synthesis in mammary gland explants from midpregnant mice (9). In addition, cyclic GMP also may contribute to the mechanism whereby prolactin stimulates casein synthesis since preincubation of mammary gland explants with cyclic GMP attenuates the time of onset of the prolactin stimulation of casein synthesis (11). Although we have measured levels of the cyclic nucleotides in explants incubated with prolactin, we have thus far been unable to detect significant changes. This, however, may be due to the fact that this tissue contains several cell types and only a minority of these are responsive to prolactin.

In any case, the elevated activity of guanylate cyclase (1) following parturition is compatible with the observation that levels of cyclic GMP are also elevated at that time. The data in Tables II and III suggest, however, that the precipitous fall in the tissue concentration of cyclic AMP following parturition cannot be explained by either an elevated activity of adenylate cyclase or a reduced activity of cyclic AMP phosphodiesterase. Nevertheless, it is possible that the elevated level of cyclic GMP may be responsible for the reduced level of cyclic AMP since Beavo *et al.* (7) have shown that cyclic GMP stimulates cyclic AMP phosphodiesterase. We have similarly found that the activity of cyclic AMP phosphodiesterase in broken cell preparations of mammary glands from midpregnant mice is doubled when either 1 or 10 μM GMP is present in the reaction tubes.

Although changes in levels of cyclic nu-

cleotides and their associated enzymes have been observed in mammary glands during pregnancy and lactation, the interpretation of these findings is complicated by the fact that the proportion of different cell types also changes during these physiological states. Also, variable amounts of milk were present in the tissues taken from lactating animals. These factors must therefore be considered when interpreting the data in these experiments.

Summary. In the mammary glands of mice, levels of cyclic AMP increased during pregnancy and then fell precipitously following parturition. In contrast, levels of cyclic GMP fell during the gestation period and then rose rapidly during the early days of lactation. Adenylate cyclase and cyclic AMP phosphodiesterase activities were elevated during the pregnancy and lactation periods.

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1. Smith, R. D., and Rillema, J. A., Proc. Soc. Exp. Biol. Med. **150,** 759 (1975).
2. Sapag-Hagar, M., and Greenbaum, A. L., Eur. J. Biochem. **47,** 303 (1974).
3. Sapag-Hagar, M., and Greenbaum, A. L., Fed. Eur. Biochem. Soc. Letters **46,** 180 (1974).
4. Steiner, A. L., Kipnis, D. M., Utiger, R., and Parker, C., Proc. Nat. Acad. Sci. USA **64,** 367 (1969).
5. Steiner, A. L., Pagliari, A. S., Chase, L. R., and Kipnis, D. M., J. Biol. Chem. **247,** 1114 (1972).
6. Krishna, G., Weiss, B., and Brodie, B. B., J. Pharmacol. Exp. Ther. **163,** 379 (1968).
7. Beavo, J. A., Hardman, J. G., and Sutherland, E. W., J. Biol. Chem. **245,** 5649 (1970).
8. Murad, F., Manganiello, V., and Vaughan, M., J. Biol. Chem. **245,** 3352 (1970).
9. Rillema, J. A., Hormon. Met. Res. **7,** 45 (1975).
10. Sapag-Hagar, M., Greenbaum, A. L., Lewis, D. J., and Hallowes, R. C., Biochem. Biophys. Res. Comm. **59,** 261 (1974).
11. Rillema, J. A., Biochim. Biophys. Acta, in press.
12. Loizzi, R. F., de Pont, J. J., and Bonting, S. L., Biochim. Biophys. Acta **392,** 20 (1975).

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