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Cyclic Nucleotides and Protein Phosphorylation in Mouse Mammary Glands: Effects of Estrogen and Progesterone Administered *in Vivo*[1] (42546)

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Abstract. Ovariectomized mice were injected daily for 20 days with saline, 17β-estradiol (1 μg/day), progesterone (1 mg/day), or estrogen + progesterone. Mammary glands were removed, homogenized, and analyzed for DNA, cAMP, cGMP, cAMP-dependent protein kinase (kinase A), cGMP-dependent protein kinase (kinase G), tyrosyl kinase (kinase T), and epidermal growth factor-stimulated tyrosyl kinase (EGF-T). Estrogen and progesterone, administered singly, increased DNA, cAMP, kinase A, kinase T, and EGF-T. In addition, progesterone, administered alone or with estrogen, decreased kinase G activity. cGMP concentrations were not altered by estrogen or progesterone. No evidence of a synergism between estrogen and progesterone on the levels of the cyclic nucleotides and the activities of kinase enzyme was observed, although an additive effect of these steroids was seen. These data indicate that ovarian steroid-induced growth of mouse mammary glands is accompanied by significant changes in protein phosphorylation, i.e., increased cAMP-dependent protein phosphorylation and tyrosyl phosphorylation and decreased cGMP-dependent protein phosphorylation.

Protein phosphorylation has been implicated in the control of a variety of cellular processes, including growth and differentiation (1). Cyclic 3′,5′-adenosine monophosphate (cAMP), one regulator of protein phosphorylation, increases during pregnancy in the mammary glands of rats, mice, and guinea pigs (2–7). Mammary cAMP-dependent protein kinase (kinase A) increases during pregnancy in rats and mice (8–10). Agents which increase intracellular cAMP concentrations (e.g., dibutyryl cAMP, cholera toxin) increase the growth of normal mammary cells *in vitro* and *in vivo* and inhibit milk component synthesis (11–20). Thus, cAMP and kinase A appear to have an important role in mammogenesis. However, the effects of individual hormones and hormone combinations on mammary cAMP levels and kinase A activity are not known.

Cyclic 3′,5′-guanosine monophosphate (cGMP) concentrations decrease during pregnancy and increase during lactation in rats and mice (3, 5). This led Sapag-Hagar and Greenbaum (5) to propose that low concentrations of cGMP were conducive to mammary growth while high cGMP concentrations were a factor in initiating lactation. Unlike kinase A, there have been no reports of cGMP-dependent protein kinase (kinase G) in mammary tissue.

Recently, a unique tyrosine phosphorylating activity was identified (21, 22). Tyrosine protein kinase activity appears to be associated with oncogene products (e.g., P60 src protein) and growth factor receptors (e.g., insulin receptor, epidermal growth factor receptor) (23). Receptors for epidermal growth factor have been identified in mammary tissue (24), but tyrosine protein kinase activity of mammary tissue has not been studied.

The objective of this study is to further define the role of protein phosphorylation in mammary gland development by examining the effects of *in vivo* treatment with 17β-estradiol and/or progesterone on cyclic nucleotide levels and protein kinase activities in mouse mammary glands.

Materials and Methods. *Chemicals.* Radioimmunoassay kits for cAMP and cGMP and [^{32}P]adenosine triphosphate (>3000 Ci/mmole) were purchased from Amersham, Arlington Heights, Illinois. Hormones and other chemicals were purchased from Sigma Chemical Co., St. Louis, Missouri.

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Animals. Female Balb/c mice (3 months old, virgin; Charles River Laboratories, Wilmington, MA) were ovariectomized, allowed 2 weeks to recover, and then injected daily for 20 days with saline (0.9% NaCl containing 1 mg/ml gum arabic; control), 17β-estradiol (1 μg/day), progesterone (1 mg/day), or estradiol + progesterone (6 mice/treatment). Estrogen and progesterone were diluted in gum arabic and injected daily in a total volume of 0.1 ml. Mice were killed by ether overdose and the fourth and fifth (inguinal) mammary glands were removed for assays.

Cyclic nucleotide-dependent kinase assays. One mammary gland from each mouse was homogenized in Tris–HCl buffer (10 m*M*, pH 7.0, containing 5% sucrose and 1 m*M* phenylmethylsulfonyl flouride). Homogenates were centrifuged (2000*g*) and the supernatants were used in protein kinase assays. The protein content of supernatant was determined using the dye binding method of Bradford (25).

Kinase A activity was determined by a modification of previously described methods (10, 26). The assay volume was 60 μl and contained 30 m*M* phosphate buffer (pH 6.8), 1 mg/ml histone, 10 m*M* $MgCl_2$, 10^{-4} *M* [γ-^{32}P]adenosine triphosphate (~250,000 cpm/tube), and 10 μl of tissue extract. Assays were performed in the absence and presence of 10^{-5} *M* cAMP. Reactions were started by adding ATP to the assay tubes. Tubes were incubated for 10 min at 30°C and stopped by transferring 30 μl to a filter paper disk (Whatman 3MM). Filter paper disks were then placed in ice-cold 10% trichloroacetic acid (TCA) for 15 min. Disks were washed three times with 5% TCA, once with 95% ethanol, once with absolute ethanol, and once with ethyl ether (15 min each). Filter paper was dried and Cerenkov radiation measured on a Beckman LS100 liquid scintillation counter in the absence of scintillation fluid. Kinase A activity was determined based on the difference in phosphate incorporation in the absence and presence of cAMP. One unit was defined as 1 pmole phosphate incorporated into histone per minute per mg protein.

Kinase G determination was similar to that of kinase A except that cGMP was substituted for cAMP and the $MgCl_2$ concentration was 30 m*M*.

Assays for kinase A and kinase G were linear over time and the amount of added homogenate. The amounts of histone, $MgCl_2$, ATP, cAMP, and cGMP were found to give maximum kinase A and kinase G activity. In assays performed in the absence of histone, cAMP and cGMP stimulated phosphorylation was 1% or less of the values in the presence of histone. Thus, endogenous substrates did not interfere with the determination of kinase A or kinase G activity.

Tyrosyl kinase assay. Tyrosine kinase activity was determined by a modification of previously reported methods (27). The assay volume was 50 μl containing 50 m*M* Tris–HCl (pH 7.5), 50 m*M* $MgCl_2$, 1 m*M* [valine-5] angiotensin II, 10 μl mammary homogenate, and 0.1 m*M* [γ-^{32}P]adenosine triphosphate (2000 cpm/pmole). Reactions were started by adding the ATP to the reaction tubes and incubating at 36°C for 10 min. Reactions were stopped by the addition of 150 μl 3.3% TCA. Next, 10 μl of bovine serum albumin (20 mg/ml) was added, tubes were centrifuged (5000*g*), and 50 μl of supernatant was spotted onto Whatman 3MM filter paper. Filter paper was washed and Cerenkov radiation was counted as described above. Background was determined in the absence of substrate ([valine-5] angiotensin II). Tyrosyl kinase was defined as the difference between background and phosphate incorporation in the presence of substrate. Epidermal growth factor-stimulated tyrosyl kinase was determined by adding 10 ng/ml epidermal growth factor to the assay mixture. Epidermal growth factor-stimulated tyrosyl kinase was defined as the difference between phosphate incorporation into [valine-5] angiotensin II in the absence and presence of epidermal growth factor. Unit definitions were the same for kinase A. Assays for tyrosine phosphorylation were linear with respect to time and the amount of enzyme added.

DNA, cAMP, and cGMP assays. Mammary glands contralateral to those used to determine protein kinase activities were homogenized in 10% TCA and centrifuged (3000*g*). Supernatant was extracted five times with 4 vol of water-saturated ether. The aqueous phase was dried under nitrogen and resuspended in buffer (0.05 *M* Tris–HCl, pH 7.5, 4 m*M* EDTA). Levels of cAMP and cGMP were de-

termined by radioimmunoassay using a commercially available kit (Amersham). Samples were diluted so that controls were expected to contain 2–4 pmole cAMP or 1–2 pmole cGMP per assay tube (50 μl for cAMP, 100 μl for cGMP; based on previous results). In addition, a second dilution (50% of the first) was assayed. Both dilutions of a sample were assayed in duplicate. Dilution of samples did not affect results. Extraction efficiency, estimated by adding ^{3}H-cAMP or cGMP to samples, averaged 91% for cAMP and 88% for cGMP. Extraction efficiency was not affected by treatment.

Water and lipids were extracted from TCA-precipitated materials by sequential washing with 1 *M* sodium acetate in methanol, methanol:chloroform (2:1, v/v), absolute ethanol, and ethyl ether. Resulting dry fat-free tissue (DFFT) was weighed and DNA was determined (28).

Statistical analysis. Data were analyzed by one-way analysis of variance. Preplanned comparisons were used to estimate the effects of estradiol and progesterone. The estradiol–progesterone interaction (nonadditivity of estradiol and progesterone effects) was also estimated. Significance was set at $P \leq 0.05$ (29).

Results. Mammary gland size, as measured by DFFT and DNA, was increased by both estradiol and progesterone (Table I). No significant interaction between estradiol and progesterone was observed. Estradiol increased mammary DNA by 76 μg/gland when administered alone and by 58 μg/gland in the presence of progesterone. Progesterone alone increased mammary DNA by 39 μg/gland in the presence of estrogen. There was no significant interaction between estradiol and progesterone on DNA.

TABLE I. WET WEIGHT, DRY FAT-FREE TISSUE (DFFT) WEIGHT AND DEOXYRIBONUCLEIC ACID (DNA) CONTENT OF MAMMARY GLANDS OF MICE TREATED FOR 20 DAYS WITH SALINE (CONTROL), 17β-ESTRADIOL (E), PROGESTERONE (P), OR E + P

	Wet weight (mg)	DFFT (mg)	DNA (μg)
Control[a]	134 ± 6	3.9 ± 0.1	247 ± 9
E	151 ± 6	4.7 ± 0.1[b]	323 ± 16[b]
P	167 ± 5[b]	4.7 ± 0.3[b]	304 ± 12[b]
E + P	180 ± 6[b]	5.4 ± 0.4[b]	362 ± 14[b]

[a] Six mice per treatment.
[b] Significantly greater than control, $P < 0.05$.

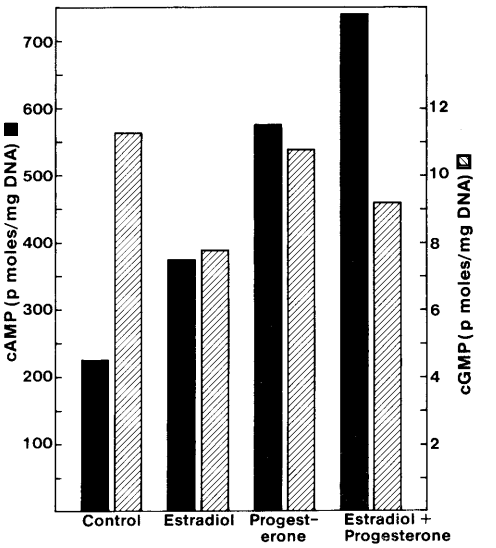

FIG. 1. Effect of 17β-estradiol and/or progesterone on cAMP and cGMP levels of mouse mammary glands. Note the different scales for cAMP and cGMP. Pooled standard error = ±45 for cAMP, ±1.3 for cGMP (six mice per treatment). Effects of estradiol and progesterone are significant ($P < 0.05$) with no significant interaction.

Estradiol increased mammary cAMP by 148 pmole/mg DNA (65% of control) in the absence of progesterone and by 164 pmole/mg DNA (72% of control) in the presence of progesterone (average of 68% of control) (Fig. 1). Progesterone increased mammary cAMP by 349 pmole/mg DNA (153% of control) in the absence of estradiol and by 365 pmole/mg DNA (160% of control) in the presence of estradiol. The effects of estradiol and progesterone together on mammary cAMP levels were additive. Mammary cGMP levels ranged from 7.8 pmole/mg DNA (estradiol-treated mice) to 11.3 pmole/mg DNA (control mice) (standard error = ±1.3). Main and interaction effects of estradiol and progesterone on mammary cGMP levels were not significantly different from zero.

Mammary kinase A activity was 135 units in controls. Estradiol increased kinase A activity of mouse mammary glands by 83 units (61%) in the absence of progesterone and by

76 units (56%) in the presence of progesterone (Fig. 2). Progesterone increased kinase A activity by 33 units (24%) in the absence of estradiol and 26 units (19%) in the presence of estradiol (average = 30 units, 22%). The interaction of estradiol and progesterone on mammary kinase A activity was not significant.

Mammary kinase G activity (Fig. 2) was decreased from 57 units in control mice and 50 units in estradiol-treated mice to 31 units in progesterone-treated mice and to 26 units in estradiol + progesterone-treated mice. The reduction of kinase G activity by progesterone was similar in the absence and presence of estradiol. Estradiol had no significant effect on mammary kinase G activity in either the absence or the presence of progesterone.

Basal mammary tyrosyl kinase activity was 14.5 units in control mice (Fig. 3). Estradiol increased basal mammary tyrosyl kinase to 29.7 units in the absence of progesterone (105% increase). Progesterone treatment resulted in basal mammary tyrosyl kinase activity of 20.0 units (38% increase). Estradiol + progesterone treatment resulted in 35.9 units (168% increase) of basal mammary tyrosyl kinase.

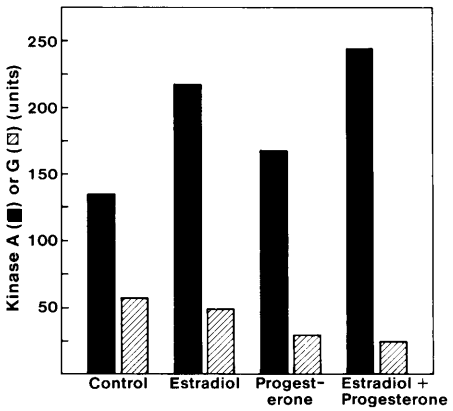

FIG. 2. Effect of 17β-estradiol and/or progesterone on cAMP-dependent protein kinase activity (kinase A) and cGMP-dependent protein kinase activity (kinase G) in mouse mammary glands. (1 unit = 1 pmole phosphate incorporated into histone per minute per milligram protein.) Pooled standard error = ±5.1 for kinase A, ±4.4 for kinase G (six mice per treatment). Effects of estradiol and progesterone are significant ($P < 0.05$) with no significant interaction.

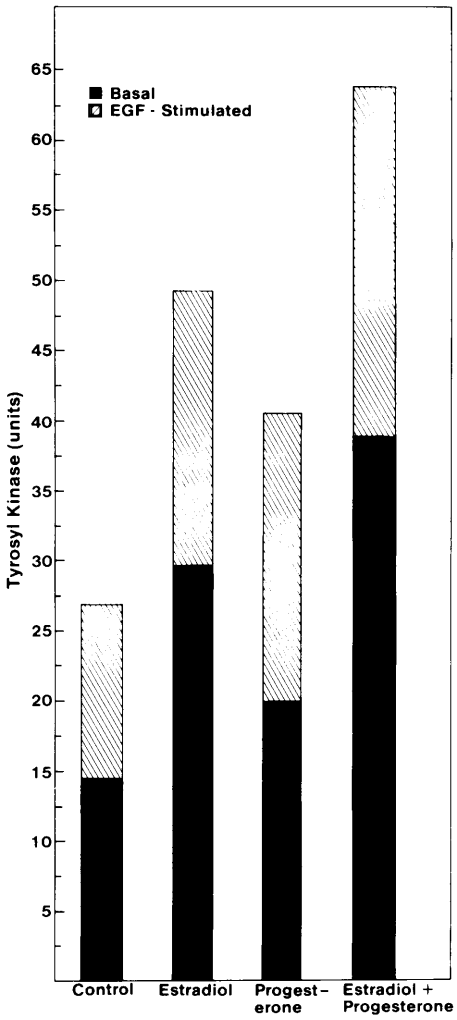

FIG. 3. Effect of 17β-estradiol and/or progesterone on basal and epidermal growth factor (EGF)-stimulated tyrosyl kinase activity in mouse mammary glands. (1 unit = 1 pmole phosphate incorporated into [valine-5] angiotensin II per minute per milligram protein.) Pooled standard error = ±1.8 for basal tyrosyl kinase, ±1.4 for EGF-stimulated tyrosyl kinase (six mice per treatment). Effects of estradiol and progesterone are significant ($P < 0.05$) with no significant interaction.

Mammary EGF-stimulated tyrosyl kinase activity (Fig. 3) was increased from 12.5 units in the control mice to 19.5 units in estradiol-treated mice, to 20.3 units in progesterone-treated mice, and to 24.7 units in mice treated with estradiol + progesterone. The main effects of estradiol and progesterone were significant

but their interaction was not significant. Insulin was unable to substitute for EGF in stimulating tyrosyl phosphorylation (data not shown).

Discussion. The results of this study indicate that estrogen- and progesterone-induced mammary growth in mice was accompanied by changes in cAMP, kinase A, kinase G, and tyrosyl kinase activity in the mammary tissue. Relative changes in these components exceeded relative changes in mammary DNA. Thus, increased mammary cAMP, kinase A, and tyrosyl kinase and decreased kinase G appeared to be due to intracellular changes and not simply due to an increased amount of epithelium.

It is generally acknowledged that adipose tissue contains little cAMP relative to most other tissues (30). Since estrogen + progesterone treatment increased the amount of epithelial tissue relative to the amount of adipose tissue in the mammary gland, we cannot eliminate the possibility that this redistribution of cell types may explain part of the observed changes in cAMP and our other measures. However, because of the magnitude of changes observed, it is unlikely that the observed changes can be attributed entirely to redistribution of cell types. For example, estrogen + progesterone increased cAMP by 212 pmole/gland. Mammary wet weight increased by 46 mg. If all of the increased wet weight were due to epithelial tissue, then the epithelial tissue would have to contain 4.6 pmole of cAMP/mg in order to explain all of the increase in mammary cAMP. Most tissues contain less than 25% of this amount of cAMP (30).

The cAMP content of mammary tissue from our control mice was slightly lower than what Rillema (3) reported for virgin mice. The mice used in the present study were ovariectomized for 5 weeks prior to cAMP determination, which probably accounts for the difference (17). Estradiol + progesterone treatment increased mammary cAMP in our study to levels similar to that observed in mice 15–16 days pregnant (3). Treatment of mice for 20 days with estrogen + progesterone results in substantial mammary ductal and lobule-alveolar development, comparable to that observed during midpregnancy (31). Thus, this experimental design (estrogen + progesterone-treated ovariectomized mice) appears to be a useful model of mammary growth, mimicking that which occurs during early and midpregnancy. These data indicate that ovarian steroids, which increase the growth of mammary glands, also increase mammary cAMP. These results are consistent with findings that cAMP increases during pregnancy (3), but the cAMP concentration attained in this study was lower than the maximum observed in mouse mammary tissue during pregnancy, which occurred during late (i.e., 18 days) pregnancy (3). Although the relative increase in mammary cAMP from controls to estradiol + progesterone-treated mice (225% on a DNA basis, 255% on a wet weight basis) was similar to that observed between virgin and 18-day pregnant mice (268% on a wet weight basis) (3), the fact that the ovariectomized controls used in this study had lower mammary cAMP concentrations than nonovariectomized virgins used by Rillema (3) may account for this similarity. If a control value similar to that observed by Rillema (3) were used, estrogen + progesterone would have still resulted in a detectable increase in cAMP concentration, but the relative increase would have been about 50% (on a wet weight basis). These data suggest that cAMP may play a role in relatively early stages of mammary gland development (i.e., the first one-half to three-fourths of pregnancy) in addition to a role in alveolar growth, suggested by the more dramatic increase in intramammary cAMP during the last one-fourth of pregnancy.

The hypothesis that the increase in intramammary cAMP observed during pregnancy or upon estradiol + progesterone treatment may be causally related to the mammogenic process is supported by results of Silberstein *et al.* (17), who observed that cholera toxin (a potent activator of adenylate cyclase (32)) increased mammary end bud formation and duct growth in mice. Agents that increase intracellular cAMP concentrations also increase *in vitro* growth of normal mammary epithelia (20). Recently, Sheffield *et al.* (16) found that injection of cholera toxin increased mammary development in mice. Cholera toxin was found to be mammogenic in ovariectomized mice and in hypophysectomized mice (in the pres-

ence of exogenous estradiol and progesterone). Cholera toxin also increased the growth of bovine mammary tissue transplanted to athymic mice (33).

The physiological effects of cAMP are generally thought to be mediated via cAMP-dependent protein kinase (kinase A). In the absence of cAMP, kinase A exists as a tetramer consisting of two catalytic subunits and two regulatory subunits (34). cAMP activates the enzyme by binding to the regulatory subunits and causing the tetramer to dissociate. This dissociation frees active catalytic subunits, which transfers the phosphate of adenosine triphosphate to a serine or threonine residue of a substrate protein. This phosphorylation alters the physiological activity of the substrate protein.

Kinase A activity of rat and mouse mammary tissue has been shown to increase during pregnancy (8–10). Unlike cAMP which continues to increase throughout pregnancy, kinase A activity increases during early to mid-pregnancy but does not increase any further during late pregnancy. Apparently, hormones of pregnancy increase the synthesis of inactive kinase A. Inactive kinase A is activated by cAMP, the concentration of which is also increased by the hormones of pregnancy, resulting in alterations of cell function (e.g., increased cell proliferation). Although such conclusions can be drawn from previous studies on pregnancy in animals, the present study provides the first evidence that *in vivo* treatment with mammogenic hormones (estradiol and progesterone) increases the amount of kinase A in mammary tissue.

cGMP concentrations in mammary glands in this study were similar to previously reported values (3). Estradiol and progesterone had no significant effects on mammary cGMP levels. It has been reported that the cGMP content of rat and mouse mammary glands decrease during pregnancy (23, 25). However, these changes were relatively small. Our results do suggest that ovarian steroids, particularly estradiol, may decrease cGMP, but this decrease did not reach the 5% level of statistical probability.

Like cAMP, cGMP is believed to exert its physiological effects through protein phosphorylation (34). cGMP-dependent protein kinase (kinase G) exists as a dimer. The enzyme is activated by the binding of cGMP. Unlike kinase A, the subunits of kinase G are not dissociated upon activation (34). To our knowledge, no studies have been conducted examining kinase G levels in mammary tissue. The results of our study indicate that progesterone decreases the kinase G content of mouse mammary glands. Sapag-Hagar and Greenbaum (5) suggested that cGMP (and, presumably, related enzymes) may be involved in initiating lactation. The decrease in kinase G may explain, in part, the inhibition of lactogenesis by progesterone.

Most protein kinases phosphorylate serine and/or threonine residues. In recent years, protein kinases that phosphorylate tyrosine residues have been identified (23). Early studies (21) indicated that tyrosyl kinase activity was associated with viral oncogene products. More recently, receptors for insulin, epidermal growth factor, and platelet-derived growth factor have been shown to possess tyrosyl kinase activity (23, 35, 36). These findings implicate tyrosyl phosphorylation as an important factor in growth control. The results of our study indicate that mammary tyrosyl phosphorylation increases during estrogen- and progesterone-induced mammary growth. The addition of epidermal growth factor to mammary gland homogenates further increased tyrosine phosphorylation, indicating that at least part of the tyrosyl kinase activity was associated with epidermal growth factor receptor. Receptors for epidermal growth factor have been identified in mammary tissue (24) and shown to increase during early pregnancy (36). Further, epidermal growth factor has been shown to have mammogenic activity (37). Thus, estrogen and progesterone may increase the responsiveness of mammary tissue to epidermal growth factor.

In summary, estrogen- and progesterone-induced mammary growth was accompanied by changes in mammary cyclic nucleotides and protein phosphorylation. Both estrogen and progesterone increased mammary cAMP, kinase A, and tyrosyl phosphorylation. In addition, progesterone decreased kinase G activity. When estrogen and progesterone were ad-

minstered together, an additive effect on cyclic nucleotides and protein phosphorylation was often seen; no evidence of a synergistic activity of these steroids, however, was observed in these studies.

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