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Hormonal Regulation of Protein Kinase C in the Mouse Mammary Gland[1] (42429)

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Abstract. The hormonal regulation of protein kinase C (PKC) induction over 3 to 14 days was investigated in the mouse mammary gland *in vitro* and *in vivo*. In intact mice, estradiol (1 μg/mouse injected daily for 2 weeks) stimulated PKC activity 70%, while progesterone (1 mg/mouse injected daily) inhibited it by 30%. Prolactin, whose levels were elevated for 2 weeks by two pituitary isografts, had no effect. When mammary gland explants were cultured in insulin and cortisol, the further addition of estradiol (1 ng/ml), progesterone (1 μg/ml), or prolactin (1 μg/ml) did not alter PKC activity after 3 days. These data suggest the following conclusions: although previous studies have implicated prolactin in the transient, calcium-phospholipid activation of PKC, it does not appear to elevate total levels of this kinase over prolonged periods. In contrast, the sex steroids do appear to affect long-term levels of this kinase; furthermore, this latter effect may be indirect.

There are numerous studies suggesting a role for protein kinase C (PKC) in mouse mammary gland development. Some have implicated PKC in epithelial growth (1), while others have argued for an involvement in differentiation (2). These seemingly contradictory claims are probably due to differences in culture conditions, since investigations in other systems show that PKC can induce either growth or differentiation, depending upon the incubation parameters (3).

Recently, this laboratory has reported that PKC activity in the mouse mammary gland is high during pregnancy but very low during lactation (4). Because mammary gland development is under hormonal control (5) and because the phosphoinositide–PKC system is activated by several hormones (6), some of which also affect the mammary gland, it seemed reasonable that this PKC profile might be the result of hormonal fluctuations during this period. In particular, since estrogens, progesterone, and prolactin are elevated during pregnancy and/or lactation (7–9), it was the purpose of this project to investigate the possible *in vivo* and *in vitro* control of PKC activity by these hormones.

Materials and Methods. Ovine prolactin (oPRL-15) was kindly provided by the Hormone Distribution Program, NIADDK (Bethesda, Md.) and crystalline porcine insulin (Lot 615-08E-220) was a gift from Eli Lilly Company (Indianapolis, Inc.). Cortisol, estradiol, progesterone, ATP, histones (lysine-rich), phosphatidylserine, and sesame oil were purchased from Sigma Chemical Company (St. Louis, Mo.). Medium 199 with Hanks' salts was obtained from Grand Island Biological Company (Grand Island, N.Y.) and [γ-^{32}P]ATP (>4000 Ci/mmol) was from ICN (Irvine, Calif.).

Virgin mice (C3H/HeN) were purchased from the National Cancer Institute (Bethesda, Md.). For the *in vivo* experiments, the mice were treated for 2 weeks with estradiol, progesterone, or prolactin. The steroids were dissolved in sesame oil and 50 μl was injected sc daily (estradiol, 1 μg/mouse; progesterone, 1 mg/mouse). Hyperprolactinemia was achieved by transplanting two pituitaries from isogenic donors under the renal capsule of each recipient animal. Controls were injected with vehicle alone or subjected to sham operations. For the *in vitro* experiments, mice were sacrificed by cervical dislocation and explants were sterilely prepared from the fourth pair of mammary glands as previously described (10). Explants were cultured on siliconized lens paper in medium 199 containing 20 m*M* Hepes (pH 7.6). All hormones, when present, were used at a concentration of 1 μg/ml except for estradiol, which was used at a concentration of 1 ng/ml. Steroids were added in absolute

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ethanol; the solvent never exceeded 0.1% (v/v) of the medium and was added to the appropriate controls. The tissue was incubated under air at 37°C and the medium was changed daily.

For the determination of the PKC activity, fresh glands or explants were homogenized in 3 vol (w/v) of 20 m*M* Tris-HCl (pH 7.5), 2 m*M* EDTA, and 50 m*M* β-mercaptoethanol and assayed by the method of Kuo *et al.* (11). Briefly, the reaction mixture contained 50 μl of whole homogenate, 5 μmole Tris-HCl (pH 7.5), 2 μmole $MgCl_2$, 40 μg lysine-rich histones, 20 nmole $CaCl_2$, 10 μg phosphatidylserine, 8 μmole NaF, and 2 nmole [γ-^{32}P]ATP ($\sim 1 \times 10^6$ cpm) in a final volume of 0.2 ml. The reaction was incubated for 5 min at 30°C and stopped by precipitation (11). Parallel reactions without calcium and phosphatidylserine were run in order to subtract out the non-PKC kinase activity. This technique has been validated for use with mammary tissue (4). Because mammary epithelium does not proliferate in explant culture (12), these results were only adjusted for the wet weight of the tissue. However, 2 weeks of hormonal treatment *in vivo* can cause epithelial growth, and the results of these latter experiments were adjusted for actual epithelial protein (13, 14). Statistical evaluations were performed by the analysis of variance.

Whole mounts of mammary glands were prepared as previously described (15).

Results. In intact mice, 2 weeks of estradiol treatment resulted in a 70% stimulation of PKC activity in the mammary gland, while progesterone injections led to a 30% inhibition. Both of these difference were observed in all treated animals and were highly significant (Table I). On the other hand, prolactin had no effect. Anatomically, the pituitary isografts were intact at autopsy; functionally, hyperprolactinemia was confirmed by examining whole mounts of the thoracic glands. Glands from sham-operated mice exhibited a simple ductal system with few alveoli (Fig. 1A); this pattern is typical for glands from virgin mice. However, glands from the animals receiving the pituitary isografts showed a more pronounced development of the epithelium (Fig. 1B), demonstrating the functional integrity of the isografts.

In the intact mouse, the possibility of hormonal interactions or indirect effects cannot be eliminated. Therefore, the effects of estradiol, progesterone, and prolactin were reevaluated *in vitro*, using the mammary gland explant culture. In this system, none of these hormones were able to affect PKC activity (Table II). All of these cultures contained cortisol at a concentration of 1 μg/ml. Because this concentration has been reported to inhibit α-lactalbumin accumulation in mammary gland explants (16), these experiments were repeated using cortisol at a much lower concentration (10 ng/ml), but the results were not different (data not shown).

TABLE I. THE EFFECT OF HORMONE TREATMENT ON PROTEIN KINASE C (PKC) ACTIVITY IN THE MOUSE MAMMARY GLAND *IN VIVO*

Treatment[a]	PKC activity (pmol [^{32}P] incorporation/g protein · min)[b]	*P*
Control	348 ± 32	—
Estradiol	590 ± 103	<0.001
Progesterone	239 ± 58	<0.005
Prolactin	362 ± 32	N.S.

[a] Mature virgin mice were treated for 2 weeks with either estradiol (1 μg daily), progesterone (1 mg daily), or prolactin (two pituitary isografts).

[b] Results are expressed as the means ± SE of five determinations.

Discussion. PKC activity can be modulated in two ways: (i) enzyme induction or (ii) allosteric activation by calcium and phospholipids. By adding calcium and phospholipids to the homogenates, PKC can be maximally stimulated; such activity correlates best with total enzyme concentration and would reflect enzyme induction. The measurement of allosteric activation is accomplished by assaying PKC in membrane fractions, since this enzyme associates with membranes after endogenous activation (17). Because mammary gland development requires a prolonged period of time to complete and because the allosteric activation of enzymes can be transient, this study concentrated exclusively on PKC induction, since this type of modulation should have a more sustained effect.

In vivo, estradiol significantly stimulated, and progesterone inhibited, PKC activity. Although an association between progesterone and PKC activity has not been previously noted, a positive statistical relationship between PKC activity and estrogen receptors has

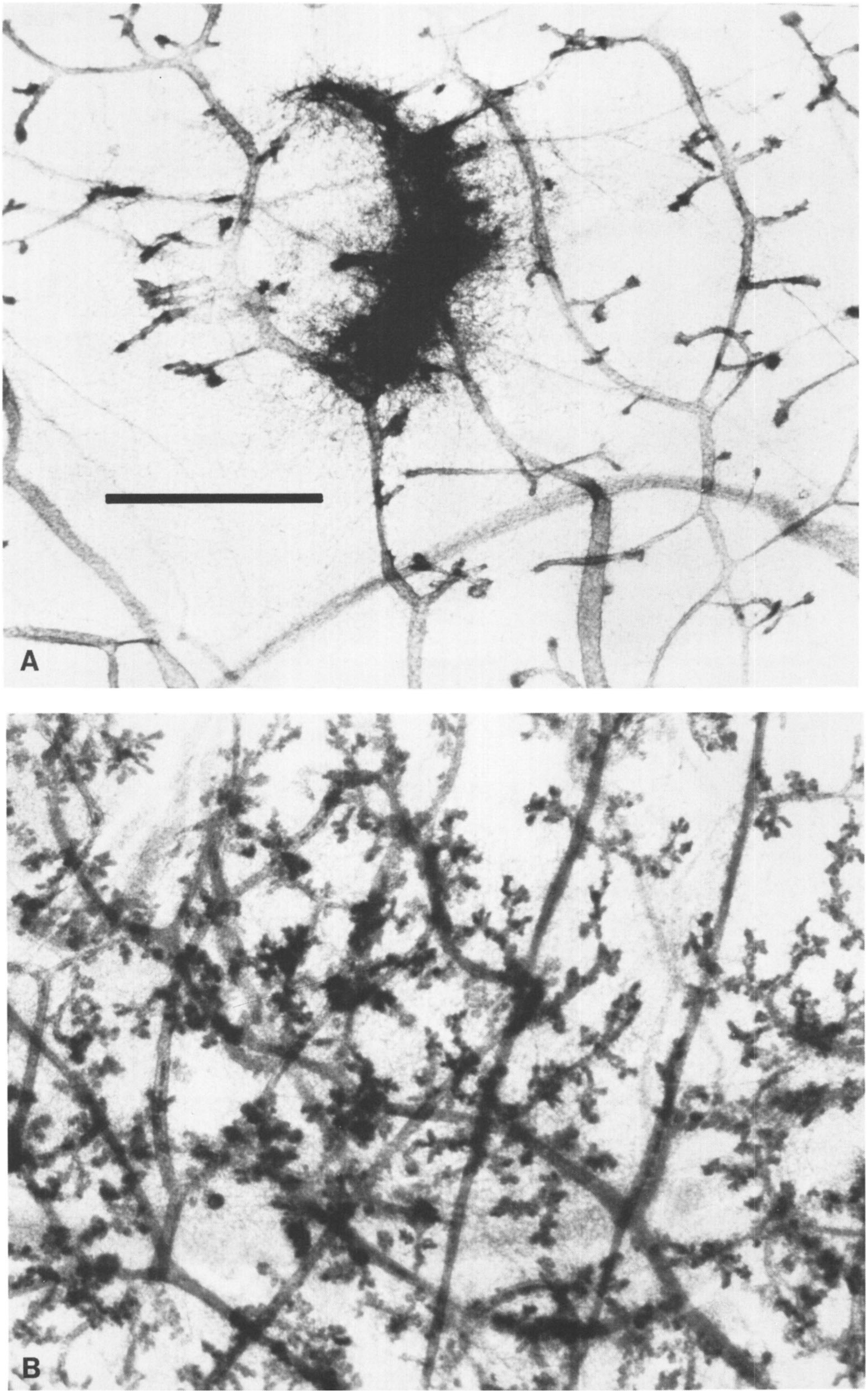

FIG. 1. Typical whole mounts of the thoracic mammary glands of mice who 2 weeks previously were either sham operated (A) or received two pituitary isografts under the renal capsule (B). The bar represents 1.0 mm.

TABLE II. THE EFFECT OF HORMONES ON PROTEIN KINASE C (PKC) ACTIVITY IN MOUSE MAMMARY GLAND EXPLANTS

Incubation conditions[a]	PKC activity (pmol [^{32}P] incorporated/g wet tissue · min)[b]
Time zero	10,694 ± 753
Insulin	9,743 ± 521
Insulin + cortisol	10,512 ± 624
Insulin, cortisol + prolactin	9,538 ± 620
Insulin, cortisol + estradiol	10,220 ± 529
Insulin, cortisol + progesterone	9,841 ± 616

[a] Mammary gland explants from virgin mice were cultured for 3 days in medium 199 containing the above hormones. The hormone concentrations were as follows: insulin, 1 μg/ml; cortisol, 1 μg/ml; prolactin, 1 μg/ml; estradiol, 1 ng/ml; progesterone, 1 μg/ml.

[b] Results are expressed as the means ± SE of 8 (steroids) to 14 determinations (prolactin). None of the means differs significantly from any other.

been reported for breast cancer cell lines (18). Unlike the steroids, prolactin had no effect on PKC induction.

In vitro, all of the hormones examined were without an effect; there are several possible explanations for these results. In the case of prolactin, it would appear that this hormone simply does not affect PKC induction, even though its stimulation of calcium fluxes (19) and phospholipid turnover in mammary explants (20) strongly suggest a role for prolactin in the allosteric activation of PKC. This transient effect may explain why prolactin must be present throughout lactation, when many of the other hormones are less important (21).

The lack of an *in vitro* response to the steroids is more puzzling since they clearly affected PKC induction *in vivo.* It is possible that these hormones affect the mammary PKC activity indirectly through some other organ or tissue. Such mechanisms have already been postulated for some of the actions of estrogen in the mammary epithelium and may involve either the generation of an estrogen intermediate (estromedin) in nonmammary tissues (22) or the neutralization of a circulating inhibitor of estrogen action (23). The mechanism of action of progesterone in mammary epithelium is less well studied but could be similar to that for estrogen.

Peptide hormones are capable of producing both acute (e.g., changes in enzyme activities) and prolonged effects (e.g., gene induction). Classically, steroids are only able to induce genes; although nongenomic effects of steroids have been suggested (24, 25), this mechanism is still controversial. Therefore, it was not surprising that the sex steroids affected total levels of PKC activity, although the apparent indirect nature of this effect was unexpected. This work further suggests that if prolactin does alter PKC activity, it must do so acutely. As such, the sex steroids may adjust the "set point" for part of the phosphoinositide pathway, while prolactin may "pull the trigger."

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