Effects of the Protein Phosphatase Inhibitor Okadaic Acid on the Actions of Prolactin in Cultured Mouse Mammary Gland Explants

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Abstract. **The effects of okadaic acid on ornithine decarboxylase (ODC) activity and milk product formation were studied in cultured mammary tissues derived from midpregnant mice. Okadaic acid stimulated ODC activity as well as the synthesis of lactose, lipids, and casein-rich phosphoproteins. When tested together, okadaic acid and prolactin evoked a nonadditive stimulation of lactose synthesis. In addition, the time-courses for the okadaic acid and prolactin effects on lactose synthesis were the same. In contrast to the effects of okadaic acid and prolactin on lactose synthesis, these agents generated additive stimulatory effects on ODC activity and the synthesis of lipids and casein-rich phosphoproteins. Since okadaic acid is known to inhibit protein phosphatases-1 and 2A, we conclude that the substrate(s) of these phosphatases are involved in the signal transduction pathway by which prolactin expresses its effect on lactose synthesis in mammary tissues. Whether these substrates are also involved in other actions of prolactin in mammary tissues remains to be established.** [P.S.E.B.M. 1993, Vol 203]

Okadaic acid is a polyether derivative of a 38-carbon fatty acid. It is a potent tumor promoter that is not an activator of protein kinase C (1), but is a powerful inhibitor of protein phosphatase-1 and phosphatase-2A (2, 3). Okadaic acid can readily penetrate the plasma membrane and it is thus useful in evaluating the possible intracellular functional roles of substrates of protein phosphatases-1 and 2A. In previous studies it has been shown that okadaic acid can cause a marked hyperphosphorylation of numerous cellular proteins, with the consequent alteration of carbohydrate and lipid metabolism in a manner consistent with its acting as a protein phosphatase inhibitor (4). Okadaic acid was employed in the present studies to determine if proteins phosphorylated on specific serine/threonine residues may be involved in the prolactin (PRL) regulation of differentiative processes in the mammary gland.

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Materials and Methods

Midpregnant (10–14 days of pregnancy) Swiss-Webster mice were purchased from Harlan Laboratories, Inc. (Indianapolis, IN). Ovine PRL (oPRL-19; 31 IU/mg) was a gift from NIAMDD. Other materials were purchased from the following sources: cortisol from Charles Pfizer and Co. (New York, NY); Medium 199 Earle's salts from GIBCO Laboratories (Grand Island, NY); porcine insulin, penicillin, and streptomycin from Eli Lilly Co. (Indianapolis, IN); [4,5-^{3}H]-leucine (53 Ci/m*M*), [1-^{14}C]-acetate (59.0 m*M*/mmol), [5,6-^{3}H]-glucose (66.6 Ci/mmol), and [1-^{14}C]-ornithine (42.5 mCi/mmol) from New England Nuclear Corporation (Boston, MA); cellulose TLC plates and a-propranol from Fisher Chemical Co. (Waltham, MA), and okadaic acid from LC Services Corporation (Woburn, MA).

The mice were killed by cervical dislocation, and the caudal pair of mammary glands were removed and placed in medium 199 Earle's salts. Explants (3–5 mg each) were prepared as described earlier (5). Explants from 6–8 animals were randomly placed on siliconized

lens papers floating on 6 ml medium 199 Earle's salts containing 1 mg/ml insulin plus 10^{-7} M cortisol. The tissues were initially cultured for 24–36 hr. at 37°C in a culture oven gassed with 5% CO_2: 95% air. All incubations were carried out in 60 × 15 mm plastic petri dishes for the lactose, lipid and casein experiments. After the initial 24–36 hr. culture period, prolactin and/or okadaic acid were added to selected plates, and incubations were continued for 8 or 10 hr. For the experiments where the rate of lactose synthesis was determined, the mammary gland tissues were exposed to 0.5 mCi/ml [5,6-^{3}H]-glucose for the last 2 hr of incubation for the pulse labeling of lactose. Labeled lactose was separated from the labeled glucose on TLC plates and the radioactivity quantitated (6). For the experiments where the rate of lipid synthesis was determined, the tissues were exposed to [^{14}C]-acetate (0.5 mCi/ml) for the final 2 hr of incubation. The tissue was then weighed, homogenized in 0.4 ml distilled water and the lipids extracted by the method of Bligh and Dyer (7). Radioactivity in the organic layer was then determined. For the experiments in which the rate of casein-rich phosphoprotein synthesis was determined, [^{3}H]-leucine (0.5 mCi/ml) was added to the culture medium for the final 2 hr of incubation. The extent of [^{3}H]-leucine was incorporated into a casein-rich phosphoprotein fraction which isoelectrically precipitated at pH 4.6 (8). For the determination of ODC activity, mammary gland tissues were preincubated for 24 hr. After preincubation, okadaic acid and/or PRL were added and incubations were continued for 4 hr. Tissue were homogenized 1:10 (wt/vol) in 50 m*M* Tris buffer (pH = 7.7). The homogenates were then centrifuged at 2000 rpm for 5 min. The supernatant was mixed with buffer containing 0.5 mCi/ml [^{14}C]-ornithine and ODC activity was assessed via the rate of [^{14}C]-CO_2 generated as described in detail earlier (9).

Statistical comparisons in these studies were made with Student's *t* test or an ANOVA followed by Scheffe's test. The statistical analyses were performed on individual replicates within experiments.

Results

Table I shows the results of two experiments in which the effects of various concentrations of okadaic acid and/or prolactin on the rate of [^{3}H]-glucose incorporation into lactose were determined. By itself, at concentrations between 0.025 and 20 n*M*, okadaic acid effected an enhanced rate of lactose synthesis. These effects were nonadditive to the response elicited by the hormone PRL. It was also possible in the experiments shown in Table I to determine the effect of okadaic acid and/or PRL on the rate of accumulation of [^{3}H]-glucose in the cultured mammary cells (data not presented). On the TLC plates on which the lactose was isolated, the areas on the plates where glucose migrated were also scrapped and radioactivity determined. After a 10-hr incubation with okadaic acid and/or PRL, neither of these substances had an effect on the rate of [^{3}H]-glucose accumulation. In further studies, the time-courses for the effects of PRL and okadaic acid on lactose synthesis were determined. Figure 1 shows that the onset time for the effects of each of these substances on lactose synthesis is about 4 hr after their addition to the tissues. In addition, the magnitudes of response to PRL and 15 n*M* okadaic acid were not different at all times between 4 and 8 hr in this experiment. In some experiments (e.g., Table I) the maximum effect of okadaic acid was less than that evoked with PRL; we can offer no explanation for the differences in magnitude of response to okadaic acid in different tissue preparations.

The data in Table II were derived from an experiment in which the effect of okadaic acid on the rate of

Table I. Okadaic Acid Effect on PRL Stimulation of Lactose Synthesis

Okadaic acid (n*M*)	Lactose synthesis (dpm/10 mg)		
	Control	PRL (1 μg/ml)	*P*
Experiment 1 (10 hr incubation)			
0	194 ± 10[a]	310 ± 9	<0.05
0.1	246 ± 16[b]	341 ± 18	<0.05
5	248 ± 12[b]	327 ± 18	<0.05
10	258 ± 14[b]	339 ± 14	<0.05
Experiment 2 (8 hr incubation)			
0	164 ± 12[a]	267 ± 22	<0.05
0.025	205 ± 11[b]	249 ± 27	<0.05
0.1	210 ± 12[b]	270 ± 13	<0.05
10	193 ± 11[b]	280 ± 17	<0.05
20	211 ± 12[b]	273 ± 26	<0.05

Numbers in the table represent the mean ± SE of at least six plates of tissues.
[b] Greater than [a] with $P < 0.05$.

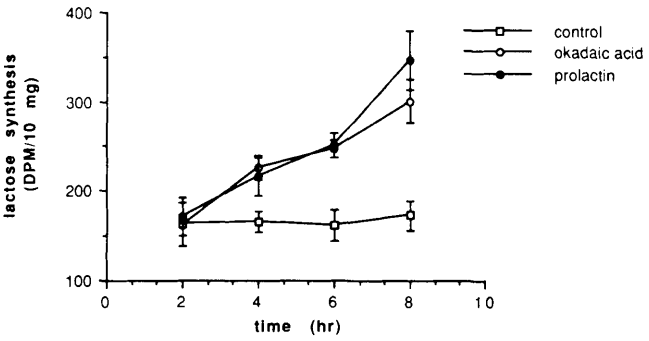

Figure 1. Time-course effects of okadaic acid (15 n*M*) and PRL (1 mg/ml) on lactose synthesis. Explants were cultured for 24 hr in the presence of 1 mg/ml insulin and 10^{-7} M cortisol. Okadaic acid or PRL was added to certain petri dishes and incubations continued for 2, 4, 6 or 8 hr. The rate of lactose synthesis was then determined as described in the text. Numbers represent the mean ± SE of at least 6 plates of tissues.

Table II. Effect of Okadaic Acid on PRL Stimulation of Phosphoprotein Synthesis

Okadaic acid (n*M*)	Casein synthesis (dpm/mg)		
	Control	PRL (1 μg/ml)	*P*
0	336 ± 9[a]	542 ± 25[c]	<0.01
0.1	360 ± 16	571 ± 22	<0.01
1	400 ± 16[b]	629 ± 19[d]	<0.01
5	402 ± 5[b]	642 ± 34[d]	<0.01
10	387 ± 8[b]	637 ± 10[d]	<0.01

Numbers in the table represent the mean ± SE of at least six pools of tissue.
[b] Greater than [a] with $P < 0.05$.
[d] Greater than [c] with $P < 0.05$.

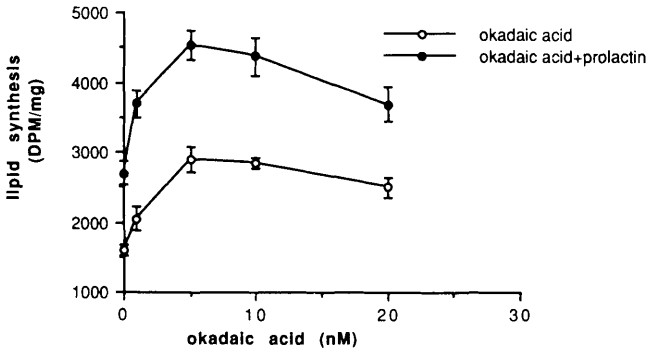

Figure 2. Effects of okadaic acid and/or PRL on the stimulation of lipid synthesis. Explants were cultured for 24 hr in the presence of 1 mg/ml insulin and 10^{-7} M cortisol. Okadaic acid and/or 1 mg/ml PRL was then added to certain petri dishes and incubations continued for 10 hr. The rate of lipid synthesis was then determined as described in the text. Numbers represent the mean ± SE of at least 6 pools of tissues.

casein-rich phosphoprotein synthesis was determined. By itself, okadaic acid at concentrations between 1 and 10 n*M* effected an increased rate of [^{3}H]-leucine incorporation into this fraction, however, each of these responses was additive to the maximum stimulatory concentration of PRL (1 μ/ml) used in these studies. A similar situation maintains (Figure 2) when the effect of various concentrations of okadaic acid and/or PRL on the rate of [^{14}C]-acetate incorporation into lipids was determined.

Finally, one of the earliest detectable effects of PRL in cultured mammary tissues is the stimulation of ODC activity. Table III shows that after a 4 hr exposure period, okadaic acid at concentrations between 1 and 200 n*M* stimulates ODC activity. Because of the variability of the data, however, with most okadaic concentrations it was not possible to statistically determine if additivity and/or synergy with the PRL response occurred. However, with 200 u*M* okadaic acid, a nonadditive response with PRL was apparent.

Discussion

These studies clearly show that okadaic acid causes PRL-like effects on the synthesis of milk product and

Table III. Effect of Okadaic Acid on PRL Stimulation of ODC Activity

Okadaic acid (n*M*)	ODC activity (dpm/10 mg)		
	Control	PRL (1 μg/ml)	*P*
0	116 ± 16[a]	426 ± 26[c]	<0.01
0.1	159 ± 24	503 ± 27	<0.01
1	202 ± 15[b]	487 ± 21	<0.01
10	195 ± 20[b]	574 ± 25[d]	<0.01
100	231 ± 25[b]	524 ± 72	<0.01
200	233 ± 11[b]	427 ± 29	<0.01

Numbers in the table represent the mean ± SE of six pools of tissues.
[b] Greater than [a] with $P < 0.05$.
[d] Greater than [c] with $P < 0.05$.

ODC activation in cultured mouse mammary tissues. Since PRL and okadaic acid elicited nonadditive effects on the rate of lactose synthesis, it would appear that these two agents are functioning via a common signal transduction pathway for regulating the rate of synthesis of this milk product. Further supporting this conclusion is the observation that the time-courses for the PRL and okadaic acid effects on lactose synthesis were the same. The results from the lactose synthesis experiments are of particular importance since it is assumed that lactose is specifically manufactured only in the PRL-responsive cells in the explanted mammary tissues. The similarity of the effects of PRL and okadaic acid on lactose synthesis thus suggests that these two substances are functioning via a common mechanism.

The effects of PRL and okadaic acid on ODC activation as well as lipid and phosphoprotein synthesis are more complex to interpret since multiple cell types in the explants are likely involved. Since fat cells constitute a large proportion of cells in the mouse mammary tissues, okadaic acid could be stimulating lipid synthesis both in the fat cells as well as the PRL-responsive cells, thus explaining the additive responses. Similarly, okadaic acid could cause the accumulation of radiolabeled phosphoproteins as well as the activation of ODC in a variety of cell types in the explanted tissues, again explaining the additivity of the okadaic acid and PRL responses. Finally, the additive effects of PRL and okadaic acid on lipid and casein synthesis could be caused by the fact that phosphatase-1 and phosphatase-2A have multiple substrates including a variety of kinases (10) and other metabolic enzymes (11, 12). The results of these studies are therefore difficult to interpret, but they are not in opposition to the thesis that prolactin expresses its effects on the synthesis of milk products in the mammary gland via a common signal transduction pathway.

Okadaic acid at concentrations between 1 and 20 n*M* effectively stimulated milk product formation and ODC activity as well. At these concentrations okadaic

acid inhibits both phosphatase-1 and phosphatase-2A. It is thus possible that the effects of PRL and okadaic acid on lactose synthesis may be carried out by the intracellular accumulation of similar phosphorylated proteins. The same may be true regarding the effects of PRL and okadaic acid on other lactogenic processes.

In previous studies we have shown that protein kinase C likely is involved in the PRL signal transduction pathway (13, 14). Since phosphatase-1 and phosphatase-2A may be involved in the dephosphorylation of the protein kinase C substrates, the fact that okadaic acid elicited PRL-like effects in mammary tissues is consistent with our earlier observations. We conclude from these and earlier studies that serine/threonine phosphorylation reactions are likely involved in the signal transduction pathway by which PRL stimulates milk product formation in the mammary gland.

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