Studies on the Possible Role of Protein Kinase C in the Prolactin Regulation of Cell Replication in Nb_2 Node Lymphoma Cells (42968)

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Abstract. **The results of several recent studies have indicated that protein kinase C (PKC) may be involved in the prolactin (PRL) stimulation of mitogenesis in the Nb_2 node lymphoma cell line. The PKC activator 12-*O*-tetradeconylphorbol-13-acetate (TPA) at certain concentrations has been shown to potentiate the mitogenic effect of PRL, whereas at higher concentrations, TPA inhibits the PRL response. Several inhibitors of PKC have also been shown to impair the PRL stimulation of metabolic process in the Nb_2 cells. These studies provide further evidence for the likely involvement of PKC in the PRL stimulation of mitogenesis in the Nb_2 cells. A transient, time-dependent accumulation of PKC in the particulate fraction of the Nb_2 cells is observed in response to PRL. TPA is also shown to elicit a similar effect, albeit at a much earlier time and with a greater magnitude. On long-term exposure (3 days), high concentrations of TPA down-regulate the PKC enzyme; this down-regulation likely accounts for the inhibitory effect of high concentrations of TPA on the PRL stimulation of cell division. In further studies, the PKC inhibitors H-7 and gossypol were shown to inhibit the PRL stimulation of cell division in a concentration-dependent fashion.** [P.S.E.B.M. 1989, Vol 192]

It has been suggested in several studies that the activation of cellular mitogenic processes may involve a regulation of protein kinase C (PKC) activity (1–3). In the Nb_2 node lymphoma cell line, the stimulation of cell division by lactogenic hormones may also involve PKC. Supporting this idea are several experimental observations. First, 12-*O*-tetradeconylphorbol-13-acetate (TPA), an activator of protein kinase C, at certain concentrations, will potentiate the mitogenic effect of a less than maximum stimulatory concentration of prolactin (PRL) (4); whereas at higher concentrations, TPA impairs the PRL stimulation of cell division. TPA also elicits PRL-like effects on ornithine decarboxylase activity as well as enhancing the rate of [^{3}H]thymidine incorporation into DNA (5, 6). In further studies inhibitors of PKC were shown to inhibit the PRL stimulation of cell division in the Nb_2 cells (7).

Other PRL-stimulated metabolic processes in the Nb_2 cells are also inhibited by PKC inhibitors; both gossypol and quercetin abolish hormone-induced increases in ornithine decarboxylase activity and the rate of [^{3}H]thymidine incorporation into DNA (8). And, finally, when phospholipase C was added exogenously to cultured Nb_2 cells, a potentiation of the mitogenic effect of a less than maximally effective concentration of PRL was observed (9). Presumably, this potentiation is carried out via the generation of diglycerides and the consequent activation of PKC.

In these studies, further evidence is presented which supports the involvement of PKC in the PRL stimulation of mitogenesis in the Nb_2 cells. Accordingly, the subcellular distribution of PKC in the Nb_2 cells in response to PRL was determined. Further studies were carried out that may explain the concentration-dependent effect of TPA in these cells. And, finally, kinase C inhibitors were employed to characterize their effect on the PRL stimulation of mitogenesis.

Materials and Methods

The Nb_2 node lymphoma cells (of rat origin) were provided by Dr. C. T. Beer of the Cancer Control Agency of British Columbia (Vancouver, British Columbia, Canada). Ovine prolactin (NIH-P-S-17) was a

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gift from the NIAMDD. Other materials used in these studies were purchased from the following sources: fetal calf serum and horse serum from GIBCO Laboratories, Grand Island, NY; gossypol, TPA, histone-type III-S, and 1-(5-isoquinolinylfulfonyl)-2-methylpiperazine (H-7) from Sigma Chemical Co., St. Louis, MO; penicillin and streptomycin from Eli Lilly Co., Indianapolis, IN; 1,2-diolein from Serdary Laboratories, Port Huron, MI; nitrocellulose membranes from Millipore Corp., Bedford, MA; DE-52 cellulose from Whatman, Clifton, NJ; disposable columns (#731-1550) from Biorad Inc., Rockville Center, NY; [γ-^{32}P]ATP (3000 Ci/mmol) from New England Nuclear/DuPont, Boston, MA.

The Nb_2 cells were maintained as suspension culture in 25-cm^2 culture flasks containing "growth medium" (Fisher's medium supplemented with 10% fetal calf serum, 10% horse serum, 1×10^{-4} *M* 2-mercaptoethanol, 50,000 IU/liter penicillin, and 50,000 μg/liter streptomycin). The cells were incubated at 37°C in the presence of a 95% air-5% CO_2 gas mixture. Fresh medium was added every 72 hr. Twenty-four hours before beginning an assay the cells were collected by centrifugation at 300*g*. The cells were then resuspended and cultured for 24 hr in "stationary medium" (same components as growth medium except for the deletion of fetal calf serum and the reduction of horse serum from 10% to 4%). The cells were next collected by centrifugation at 300*g* and used for experimentation. When the effects of PRL, H-7, TPA, and/or gossypol on cell number was to be determined, 1-ml aliquots ($1-1.5 \times 10^5$ cells/ml) were transferred to the wells of sterile multiwell tissue culture plates (24 plates/well). The cells were then cultured for an additional 72 hr in the presence of appropriate concentrations of PRL, TPA, H-7, and/or gossypol. Following this incubation the cells were transferred to vials containing 9 ml of Hematall isotonic fluid and cell number was determined on a Coulter model ZM counter. Statistical comparisons were made using an analysis of variance and Tukey's HSD procedure or a paired *t* test where appropriate.

In experiments where the effects of PRL and TPA on kinase C distribution and activity were determined, $25-30 \times 10^6$ cells were required for each experimental observation. After cells were cultured for 24 hr in the stationary medium, $25-30 \times 10^6$ cells were suspended in 10 ml of stationary medium containing the appropriate concentrations of PRL and/or TPA; the cells were cultured at 37°C in 25-ml Erlenmeyer flasks for specified times. The cells were then harvested by centrifugation at 300*g*, washed once in 20 ml of ice-cold buffer (20 m*M* Tris-HCl (pH 7.5, 4°C), 2 m*M* EDTA, 0.5 m*M* EGTA, 5 m*M* dithiothreitol, and 5 μg/ml leupeptin), and subsequently resuspended in 20 ml of this same buffer. The cells were homogenized by a 5-sec sonication. The homogenates were then centrifuged for 60 min at 100,000*g* to partition the particulate and cytosolic fractions. PKC from the cytosolic fractions and detergent-solubilized particulate fractions (1 hr at 4°C in the resuspension buffer containing 1% Nonidet-P40, and lacking the leupeptin) was partially purified by DEAE chromatography (10); PKC activity was subsequently determined. PKC activity was quantitated by measuring the incorporation of ^{32}P from [^{32}P]ATP into histone-type III-S as described by Thomas *et al.* (10). The reaction was stopped after 6 min at 30°C by the addition of 25% trichloroacetic acid; the precipitate was collected and washed on Millipore nitrocellulose filters. The filters were added to vials containing scintillation cocktail and ^{32}P was quantitated in a Packard model 4430 scintillator counter. The PKC assay was done under conditions in which reaction rate was proportional to enzyme concentration and times between 2 and 8 min at 30°C. Specific PKC activity is defined as the difference between the activity measured under "optimal" conditions (i.e., w/$CaCl_2$ and phospholipid) and that obtained with $CaCl_2$ alone. Activities are expressed as milliunits/mg protein. One international unit is that amount of enzyme which catalyzes the incorporation of 1 μmol of phosphate from ATP into histone per minute (30°C, pH = 7.5). Protein determinations were made by a modified Lowry procedure (11) using bovine serum albumin as standard.

Results

In Table I is presented the results of an experiment where kinase C activity was determined in cell fractions after Nb_2 cells were cultured with or without PRL for 16 hr. About 20% of the kinase C activity is found in the particulate fraction and 80% in the cytosolic fraction. Clearly PRL has no effect on either kinase C activity or cellular distribution of kinase C after a 16-hr exposure period. Figure 1 shows the time course for the effect of PRL on kinase C distribution in the particulate fraction. PRL enhances kinase C activity in that fraction at times between 4 and 12 hr after PRL addition. A stimulatory effect is not apparent at times prior to 4 hr or after 12 hr. PRL thus has a transient effect on altering kinase C distribution in the Nb_2 cells.

The effect of the kinase C activator TPA on Nb_2 lymphoma cell number after a 72-hr treatment is shown

Table I. Kinase C Distribution in Nb_2 Cells[a]

Fraction	Kinase C activity (milliunits/mg protein)		
	Control	PRL (50 ng/ml)	*P*
Cytosolic	0.444 ± 0.030[b]	0.446 ± 0.053	NS[c]
Particulate	0.111 ± 0.042	0.126 ± 0.026	NS
Total	0.555	0.572	

[a] Nb_2 cells were cultured for 16 hr in the presence or absence of PRL and kinase C activity was then determined in the 100,000*g* pellet (particulate) and supernatant (cytosolic) fractions.
[b] Mean ± SE of six observations.
[c] NS, not significant.

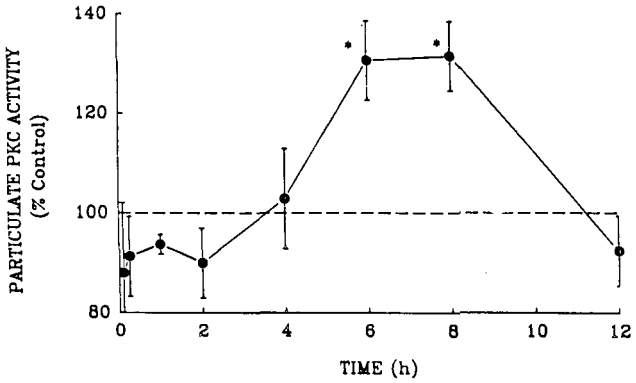

Figure 1. Time course for the effect of PRL on kinase C activity in the particulate fraction of Nb_2 lymphoma cells. Nb_2 cells were cultured plus or minus 50 ng/ml PRL for the times indicated. Kinase C activity was then determined in the 100,000*g* pellet fraction of the cellular homogenates. Each experimental value represents three to six observations.

Table II. Effect of TPA on Mitogenesis in Nb_2 Cells[a]

TPA concentration (n*M*)	Numbers of cells ($\times 10^{-3}$ after 72 hr)		
	No PRL	50 pg/ml PRL	50 μg/ml PRL
0	109 ± 1[b]	480 ± 9	771 ± 16
0.1	102 ± 2[c]	592 ± 16[d]	752 ± 21
1.0	93 ± 3[c]	552 ± 13[d]	721 ± 20
10.0	98 ± 4[c]	420 ± 10[c]	746 ± 27
100	97 ± 3[c]	326 ± 7[c]	695 ± 8[c]
1000	93 ± 3[c]	269 ± 5[c]	608 ± 9[c]

[a] Cells were cultured for 72 hr in the presence of TPA and/or PRL at the concentrations indicated. Cell number was then determined.
[b] Mean ± SE of six determinations.
[c] Less than in the absence of TPA.
[d] Greater than in the absence of TPA.

in Table II. By itself, TPA at all concentrations between 0.1 and 1000 n*M* caused a small but significant decrease in cell number. In the presence of a concentration of PRL (50 pg/ml) that elicits about a half-maximal response on mitogenesis, TPA at 0.1 and 1.0 n*M* potentiates the PRL stimulation of cell division. At higher concentrations (10–1000 n*M*), however, TPA inhibits the PRL response in a concentration-dependent fashion. When testing TPA in the presence of a maximum stimulatory concentration of PRL (50 ng/ml), the only effect observed was an inhibition of the PRL response when 100 and 1000 n*M* TPA were employed.

To clarify the complex effects of TPA on the Nb_2 cells, two additional experiments were carried out. In the first, the effect of a 6-min, 5 μM TPA treatment on kinase C distribution in the Nb_2 cells was determined. The data in Table III show that in 6 min TPA causes a shift of kinase C in the particulate fraction from 14 to 98%. Clearly, TPA is efficacious in effecting a rapid redistribution of kinase C in the Nb_2 cells. In a second experiment, the effect of a 72-hr culture with TPA and/or PRL on total kinase C activity was determined (Table IV). The data show that TPA down-regulates total kinase C activity in a concentration-dependent manner in the presence of a maximum stimulatory concentration of PRL. It is also apparent from the data in Table IV that PRL maintains kinase C activity in the Nb_2 cells over a 72-hr culture, since in the absence of PRL, kinase C activity becomes negligible.

In two final experiments (Tables V and VI), the effects of two kinase C inhibitors on the PRL stimulation of mitogenesis were determined. Both H-7 (at 0.5 and 1 m*M*) and gossypol (at 100 and 500 μM) abolished the PRL stimulation of cell division.

Table III. Effect of 6-Min TPA Treatment on Kinase C Distribution in Nb_2 Lymphoma Cells[a]

Cell fraction	Control	5 n*M* TPA
Cytosolic	0.736 (76%)	0.020 (2%)
Particulate	0.118 (14%)	0.965 (98%)
Total	0.855	0.985

[a] Nb_2 cells were incubated for 6 min with or without 5 n*M* TPA. Kinase C activity was then determined in the 100,000*g* pellet and supernatant fractions.

Table IV. Total Kinase C Activity in Nb_2 Lymphoma Cells after 72 hr with TPA and PRL (50 ng/ml)[a]

Addition	Kinase C activity (milliunits/mg protein)
—	0.0195 ± 0.0045
PRL	0.413 ± 0.06
PRL + 0.5 n*M* TPA	0.301 ± 0.027
PRL + 100 n*M* TPA	0.015 ± 0.007

[a] Values represent the results from triplicate determinations. Nb_2 cell were incubated for 72 hr with the indicated treatments. Cells were then collected, washed, and sonicated as described in the text. Nonidet P-40 was added to a final concentration of 1% (v/v) and homogenates were maintained at 0–4°C for 60 min. After DEAE chromatography, PKC activity was measured.

Table V. Effect of H-7 on PRL Stimulation of Cell Division in Nb_2 Lymphoma Cells[a]

H-7	Cell/ml ($\times 10^{-3}$)		
	Control	PRL (50 ng/ml)	*P*
0	177 ± 4[b]	502 ± 7	<0.01
100 n*M*	173 ± 2	530 ± 8	<0.01
10 μM	176 ± 4	491 ± 5	<0.01
100 μM	168 ± 3	441 ± 4	<0.01
500 μM	150 ± 7	160 ± 10	NS[c]
1 m*M*	147 ± 7	144 ± 5	NS

[a] After a 24-hr culture in stationary medium, the growth-arrested cells were harvested and resuspended in fresh stationary medium at 1.0–1.5 × 10^5 cells/ml. H-7 and/or PRL at the concentrations indicated were then added to the cells and incubation continued for 72 hr. Cell number was then determined.
[b] Mean ± SE of six observations.
[c] NS, not significant.

Table VI. Effect of Gossypol on PRL Stimulation of Cell Division in Nb_2 Lymphoma Cells[a]

Gossypol	Cell/ml ($\times 10^{-3}$)		
	Control	PRL (50 ng/ml)	*P*
0	110 ± 4	832 ± 10	<0.01
1 n*M*	109 ± 1	834 ± 8	<0.01
100 n*M*	111 ± 2	824 ± 15	<0.01
10 μM	112 ± 2	782 ± 15	<0.01
100 μM	92 ± 4	92 ± 3	NS[b]
500 μM	69 ± 4	60 ± 8	NS

[a] Nb_2 cells were cultured for 72 hr with gossypol and/or PRL at the concentrations indicated. See Table V for details.
[b] NS, not significant.

Discussion

PRL was found to induce a transient, time-dependent accumulation of kinase C in the particulate fraction of Nb_2 cells. This accumulation occurred between 4 and 12 hr after adding PRL to the cells. A similar delayed redistribution of kinase C was observed in human β lymphocytes in response to a polyclonal mitogen; a large effect was observed at 4 hr but only a small response was observed after 30 min (12). In several other cell lines, effects of mitogens on kinase C redistribution have been shown to occur within minutes after addition of the mitogens (13–15). It is not presently known why the effects of some mitogens on kinase C redistribution occur rapidly, whereas others, including the PRL effect on the Nb_2 cells, only become manifest after several hours. It is interesting to note that on a time course basis, the PRL stimulation of ornithine decarboxylase activity, *S*-adenosylmethionine decarboxylase activity, as well as the initiation of an enhanced rate of [^{3}H]thymidine incorporation into DNA (16) in the Nb_2 cells all occur in concert with the increased accumulation of kinase C in the particulate fraction, i.e. between 4 and 12 hr. Whether there is a direct cause and effect relationship between the stimulation of these processes remains to be established.

The apparently conflicting information concerning the effects of TPA on mitogenesis in the Nb_2 cells may be explained by the two effects that TPA elicits on these cells. First of all, prolonged treatment (72 hr) with a high concentration of TPA causes a down-regulation of PKC activity in the Nb_2 cells. At the same time, a concentration-dependent TPA inhibition of the PRL stimulation of cell division is observed both in the presence of an intermediate and maximum stimulatory concentration of PRL. In other cell types, prolonged treatment with TPA has also been shown to decrease PKC activity (17, 18), as well as decrease the numbers of phorbol ester binding sites (19–21), and decrease the quantity of immunoprecipitible PKC (22). It thus seems likely that down-regulation of PKC by prolonged exposure of Nb_2 cells to high concentrations of TPA may be causally related to the inhibition of the PRL-stimulated cell division. When lower concentrations of TPA were employed (0.1–1 n*M*), only a small effect on the down-regulation of PKC was observed after a 72-hr incubation. In addition, TPA at the lower concentrations was shown to potentiate the mitogenic effect of PRL at a hormone concentration that induced a less than maximum response. In the presence of a maximum stimulatory concentration of PRL, however, TPA at the lower concentrations was without effect. The results of these experiments are compatible with the conclusion that the mitogenic effect of PRL in the Nb_2 cells likely involves the participation of PKC.

Further supporting this conclusion are studies in which the PKC inhibitors H-7 and gossypol were found to abolish the PRL stimulation of mitogenesis. In an earlier study, H-7 was reported to inhibit the mitogenic effect of human PRL on the Nb_2 cells (7); however, H-7 was reported to be about 10-fold more potent than we observed in our studies. The difference may be related to the use of human versus ovine PRL. Gossypol, a second PKC inhibitor, has been shown in earlier studies to abolish the prolactin stimulation of ornithine decarboxylase activity as well as the rate of [^{3}H]thymidine incorporation into DNA in the Nb_2 cells (8). The inhibitory concentrations of gossypol employed in those studies correspond well with those that we found to inhibit the PRL stimulation of cell division. The experiments with PKC inhibitors therefore provide further evidence supporting the likely involvement of PKC in the mitogenic effect of PRL in the Nb_2 cells.

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