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Rapid activation of protein kinase C in isolated rat liver nuclei by prolactin, a known hepatic mitogen

(prolactin receptor/dose dependence/anti-prolactin antiserum inhibition/anti-prolactin receptor antibody inhibition)

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ABSTRACT **Rat liver nuclei pure by enzymatic and electron microscope criteria contain protein kinase C (PKC) that can be activated several hundredfold within 3 min of addition of prolactin or phorbol 12-tetradecanoate 13-acetate. Rat prolactin stimulated PKC maximally at 10^{-12} M, whereas ovine prolactin was maximally stimulatory at 10^{-10} M. Activation was time and dose dependent, exhibited a biphasic pattern, and was blocked by anti-prolactin antiserum, by PKC inhibitors such as 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H-7) and sphingosine, and by cyclosporine. Moreover, the ability of prolactin to activate nuclear PKC was inhibited totally by a monoclonal antibody to the rat liver prolactin receptor, implicating a prolactin receptor-mediated activation process. Epidermal growth factor (EGF), a liver mitogen, caused a lesser but significant activation of nuclear PKC. However, EGF and suboptimal prolactin were synergistic. Human growth hormone, which has lactogenic properties, stimulated PKC activity, whereas nonlactogenic substances such as ovine growth hormone, insulin, dexamethasone, and 8-bromo-cAMP were inactive. That this may be a general mechanism for prolactin is suggested by the ability of prolactin to stimulate PKC 140-fold in rat splenocyte nuclei. Prolactin has comitogenic properties in lymphocytes.**

Prolactin is an adenohypophyseal polypeptide hormone thought to exert its effects on macromolecular synthesis at the level of the cell nucleus and Golgi complex after receptor-mediated endocytosis (1, 2). Administration of prolactin to rats induces hepatic ornithine decarboxylase and plasminogen activator (3–5), specific enzyme markers expressed early during the G_1 phase of the cell cycle, and stimulates DNA synthesis and subsequent hepatomegaly (6, 7). The effects of prolactin on hepatic cell cycle progression mimic those in response to partial hepatectomy (8), a response regulated by circulating factors, as demonstrated in the classic parabiotic experiments of Moolten and Bucher (9). Tumor promoters such as phorbol 12-tetradecanoate 13-acetate (PTA) also induce ornithine decarboxylase and plasminogen activator in the liver and other organs such as the skin (10–12). Further, prolactin has tumor-promoting properties in carcinogen-exposed rat liver (7). Thus, it seemed likely that prolactin might exert its cellular action through the activation of protein kinase C (PKC).

Prolactin administration to rats results in a rapid activation of hepatic PKC with a characteristic elevation of activity in the particulate fraction (13). The direct activation of PKC by the addition of PTA (14) elevated particulate PKC activity in a manner similar to prolactin. In addition, partial hepatectomy triggered a rapid increase in circulating prolactin (within 1 min) and an elevation of PKC activity in the particulate fraction of the remaining liver (13). These data provide evidence that prolactin-dependent intracellular signaling involves the activation of PKC. Other studies have shown that PTA is capable of stimulating mitogenesis in a prolactin-dependent cell line, the Nb2 node lymphoma cell line (15, 16), and inhibitors of PKC block prolactin-stimulated proliferation in this cell line and affect prolactin-requiring processes in other systems (17, 18).

The recent characterization of the rat liver prolactin receptor by Kelly and coworkers (19) demonstrated a structure similar to that of receptors that are primarily transporters of molecules (such as transferrin). Concurrently, other workers reported nuclear PKC detected both immunochemically and by its calcium and phospholipid activity requirements in liver (20) and in NIH 3T3 cells (21). In lymphocytes, a nuclear lamina protein, lamin B, was rapidly phosphorylated after PKC activation with PTA (22).

The possibility of prolactin receptors at the nucleus (1, 2), the presence of PKC in rat liver nuclei and subnuclear fractions (20), and the ability of PKC to influence the nuclear transcription of specific genes (23, 24), including the ornithine decarboxylase gene(s) (23), led us to explore whether prolactin could activate nuclear PKC. We found a rapid several-hundredfold activation of PKC in rat liver nuclei in response to prolactin that was dose dependent and could be blocked by rat anti-prolactin receptor antibody and by anti-prolactin antiserum.

MATERIALS AND METHODS

Materials. Male Sprague–Dawley rats (125–150 g) were obtained from Harlan Sprague Dawley (Indianapolis). The polypeptide hormones ovine, rat, and bovine prolactin and human, ovine, and rat growth hormone as well as antisera to ovine and rat prolactin were obtained from the National Hormone and Pituitary Program (National Institute of Diabetes and Digestive and Kidney Diseases). Epidermal growth factor (EGF), zinc-free insulin, 8-bromo-cAMP, sphingosine, PTA, and 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H-7) were obtained from Sigma. Cyclosporine (Sandimmune) was obtained from Sandoz. The monoclonal antibody to the rat hepatic prolactin receptor (E29) was a generous gift from Paul A. Kelly (McGill University, Montreal).

Preparation of Liver Nuclei. Hepatic nuclei were prepared by using a modification of the technique of Widnell and Tata (25). Rats were killed by cervical dislocation. The liver was perfused, washed, and minced in 2 vol of ice-cold buffer A (25 mM Tris·HCl, pH 7.4 at 4°C/3 mM $MgCl_2$/0.32 M sucrose/2 mM EGTA/0.1 mM spermine/0.1% Triton X-100 and leupeptin at 50 μg/ml). The tissue was homogenized with a glass Dounce homogenizer, filtered through two layers of nylon mesh, and diluted to 0.2 M sucrose. Buffer A was layered beneath the homogenate and the sample was centrifuged at

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Abbreviations: PKC, protein kinase C; PTA, phorbol 12-tetradecanoate 13-acetate; H-7, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine; EGF, epidermal growth factor.

700 × *g* for 10 min. The pellet was resuspended in a buffer containing 2.4 M sucrose, 25 mM Tris·HCl (pH 7.4 at 4°C), 1 mM $MgCl_2$, 2 mM EGTA, 0.1 mM spermine, and 0.1% Triton X-100 and centrifuged at 50,000 × *g* for 60 min at 4°C. The nuclear pellet was washed in a modified Hanks' balanced salt solution (HBSS) containing 5 mM $MgCl_2$ and resuspended at 1–5 × 10^7 nuclei per ml. Nuclear preparations were routinely assessed for membrane contamination. These preparations had no detectable 5′-nucleotidase activity (26) and had no contamination visible with light or electron microscopy (Fig. 1).

For examination by electron microscopy, nuclear samples were incubated overnight in 2.5% glutaraldehyde in a 0.1 M sodium phosphate buffer (pH 7.4), fixed in 2% osmium tetroxide, dehydrated with acetone, and embedded in LX-112 resin (Ladd, Burlington, VT). Sectioned samples (700 nm) were stained with uranyl acetate and lead citrate prior to examination with a Phillips CM-10 transmission electron microscope (H. Lee Moffitt Cancer Center and Research Institute, Department of Pathology, University of South Florida).

Preparation of Splenocyte Nuclei. Rats were killed by cervical dislocation. The spleens were forced through a fine wire screen, rinsed in modified HBSS/0.3% bovine serum albumin, and rocked for 10 min at room temperature. The cell suspension was centrifuged at 300 × *g* for 5 min and the pellet was resuspended in a pH 8.0 buffer containing 0.2 M NH_4Cl, 10 mM potassium carbonate, and 0.1 mM EDTA, inverted once, filtered, and centrifuged at 300 × *g* for 5 min. Nuclei were isolated from splenocytes resuspended in HBSS/0.3% bovine serum albumin by using the technique described by Wolff *et al.* (27). The splenocyte suspension was centrifuged at 300 × *g* for 5 min, and resuspended at 1.5–3 × 10^7 cells per ml in a buffer containing 0.4 mM potassium phosphate at pH 6.7, 2 mM $MgCl_2$, 0.1% Triton X-100, and 0.1 mM spermine. The sucrose concentration was adjusted to 0.32 M and the cells were centrifuged at 300 × *g* for 5 min. The pellet was resuspended at 10^7 cells per ml in 0.32 M sucrose/4 mM potassium phosphate, pH 6.7/2 mM $MgCl_2$/4 mM EDTA/0.1 mM spermine/0.1% Triton X-100/1 mM phenylmethanesulfonyl fluoride/15 μg of leupeptin per ml and disrupted in a Dounce homogenizer. The sample was centrifuged at 450 × *g* for 10 min at 4°C and the nuclear pellet was resuspended in modified HBSS (10^8 nuclei per ml).

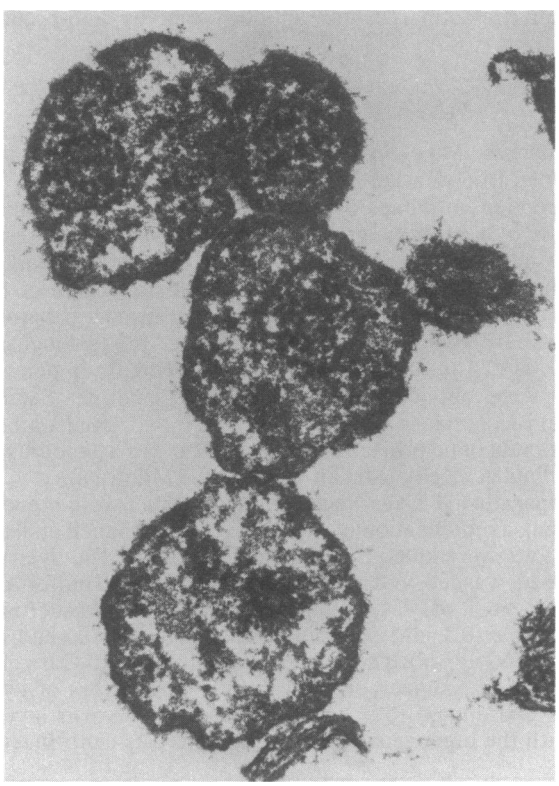

FIG. 1. Electron micrograph of a representative preparation of isolated rat hepatic nuclei. (×6800.)

Incubation of Nuclei. Nuclei were incubated in 25-ml Erlenmeyer flasks at 37°C in a shaking water bath. Calcium was added so that the final concentration was 1.0 mM. The reaction was initiated by hormone addition and terminated by the addition of 9 vol of ice-cold phosphate-buffered saline (Whittaker, Walkersville, MD) and centrifugation at 1300 × *g* for 20 min at 4°C. The nuclei were resuspended in 1 ml of buffer B (25 mM Tris·HCl, pH 7.4 at 4°C/0.25 M sucrose/0.5 mM EGTA/2 mM EDTA/2.5 mM $MgCl_2$/50 mM 2-mercaptoethanol/0.5 mM phenylmethanesulfonyl fluoride/16 μg of leupeptin per ml/0.02% Triton X-100) and disrupted by sonication (five 15-sec bursts with 5-sec rests). Purity of the nuclear preparation was verified by the absence of 5′-nucleotidase activity and by electron microscopy (Fig. 1).

PKC Assay. Nuclear PKC was determined by using a modification of the technique described by Kuo and coworkers (28) by measuring Ca^{2+} and phospholipid-dependent phosphorylation of lysine-rich histone (50 μg per assay, Sigma type III-s). The reaction in 20 mM Tris·HCl, pH 7.4/50 mM 2-mercaptoethanol/1 mM phenylmethylsulfonyl fluoride/8 mM $MgCl_2$/0.5 mM $CaCl_2$/12 μg of leupeptin per ml/10 μM ATP (containing 10^6 cpm of [γ-^{32}P]ATP) with or without phosphatidylserine at 25 μg/ml and dioleoylglycerol at 4 μg/ml was initiated by the addition of 1–10 μg of protein in a total volume of 250 μl and was terminated by the addition of 3 ml of ice-cold 25% trichloroacetic acid. Protein precipitate was collected by filtration through Whatman GF/C filters. Filters were washed twice with 3 ml of 10% trichloroacetic acid and dried and their radioactivities were measured by liquid scintillation spectroscopy. Enzyme activity was determined by subtracting the amount of ^{32}P incorporated into histone in the absence of added phospholipids from that incorporated in the presence of phospholipids.

Statistical Determinations. Statistical differences between treatments were determined by the Newman–Keuls range for multiple comparisons, by analysis of variance (ANOVA), or by the Student *t* test for unpaired data. Differences were considered significant at the 95% confidence level.

RESULTS AND DISCUSSION

Time Course for Prolactin Activation of PKC in Rat Liver Nuclei. Within 1 min after the addition of prolactin, nuclear PKC activity was increased significantly ($P < 0.01$) compared to vehicle controls (Fig. 2). Maximal prolactin activation of nuclear PKC was found within 3 min ($P < 0.01$). The time course for prolactin activation of PKC in nuclei isolated from rat liver is nearly identical to that reported by Cambier *et al.* (29) for antibodies directed against Ia antigens and cyclic nucleotides on nuclear PKC in murine splenocytes. These authors interpreted their results to reflect translocation of PKC from the splenocyte cytosol to the nucleus. However, in the present study, hepatic nuclei were prepared free of contamination from either the cytosol or membrane (Fig. 1). Therefore, it appears that prolactin activated PKC present in the hepatic nuclei.

Dose Dependency of Prolactin-Stimulated Rat Liver Nuclear PKC. Ovine prolactin at concentrations as low as 10^{-15} M caused detectable activation of PKC in rat liver nuclei (Fig. 3). Ovine prolactin at 10^{-10} M consistently resulted in the greatest activation of nuclear PKC. The activation by rat prolactin was somewhat less than ovine prolactin at 10^{-10} M. However, rat prolactin was more potent than the ovine homologue, stimulating nuclear PKC to greater than 130-fold

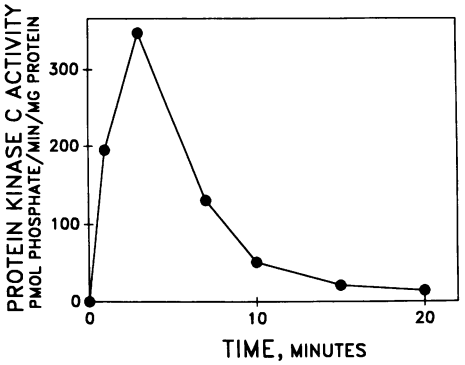

FIG. 2. Time course of prolactin stimulation of nuclear PKC activity. Rat hepatic nuclei were incubated in the presence of 10^{-10} M prolactin at 37°C for the times indicated, after which PKC was assayed in triplicate. A representative experiment is shown. This experiment was repeated three times with equivalent results. All time points, with the exception of 20 min, differed significantly ($P < 0.01$) from controls (zero-time point).

vehicle controls at 10^{-12} M. Prolactin stimulation of nuclear PKC was biphasic, a characteristic previously described for prolactin effects in other systems (30–32).

Effect of Polypeptide Hormones and Other Biological Response Modifiers on PKC Activity in Isolated Rat Liver Nuclei. The ability of various prolactins and related growth hormones to stimulate nuclear PKC was assessed (Table 1). Ovine, rat, and bovine prolactin significantly stimulated nuclear PKC. Human growth hormone, which recognizes both somatotropin and prolactin receptors in rat liver (33), also significantly stimulated PKC in the rat liver nucleus, whereas ovine and rat growth hormones were ineffective.

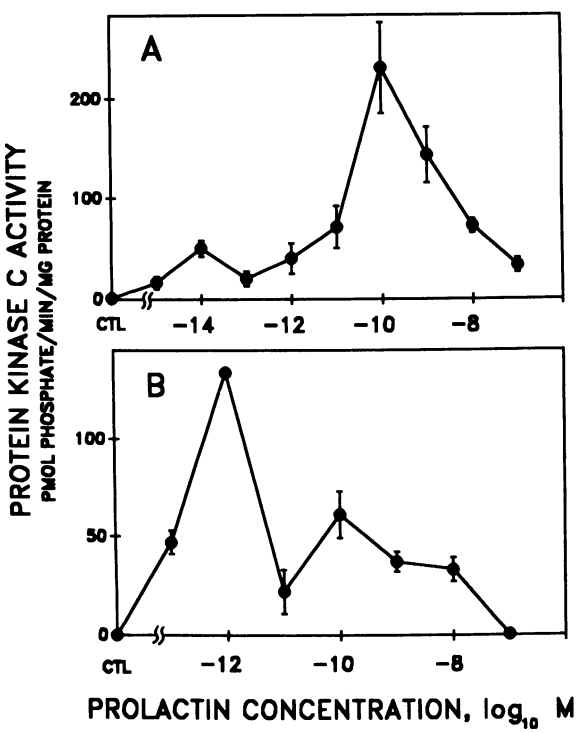

FIG. 3. Dose response for prolactin activation of PKC in rat hepatic nuclei. Dose-dependent activation of nuclear PKC in response to ovine (*A*) or rat (*B*) prolactin is presented. Rat hepatic nuclei were incubated at 37°C for 3 min in the presence of hormone at the concentration indicated on the abscissa. PKC was assayed in triplicate, and the data represent the mean ± SEM of three separate experiments. All data points differed significantly ($P < 0.01$) from the respective vehicle control (CTL) with the exception of 10^{-7} M rat prolactin.

Table 1. Effect of various hormones and biological response modifiers on PKC activity in isolated rat liver nuclei

Treatment	PKC, pmol phosphate per min per mg protein
Vehicle control	1 ± 0.8
Ovine prolactin	229 ± 46*
Rat prolactin (10^{-12} M)	134 ± 2*
Bovine prolactin	91 ± 15*
Human growth hormone	64 ± 3*
Ovine growth hormone	1 ± 0.9
EGF	37 ± 5*
Ovine prolactin (10^{-11} M)	76 ± 11*
EGF + ovine prolactin (10^{-11} M)	205 ± 21*
Insulin	5 ± 2
Dexamethasone	1 ± 0.7
PTA	201 ± 35*
8-Bromo-cAMP (10^{-5} M)	1 ± 0.6

Rat liver nuclei were incubated for 3 min at 37°C with the indicated hormone or other substance at 10^{-10} M unless otherwise specified. PKC activity was measured in triplicate. Data represent the mean ± SEM of three separate experiments. *$P < 0.01$ vs. vehicle control.

EGF also significantly stimulated nuclear PKC (Table 1). This is of particular interest since EGF is mitogenic for hepatocytes maintained in culture (34, 35). However, when EGF was added to hepatic nuclei in the presence of prolactin at a suboptimal concentration (10^{-11} M), there was a marked synergistic response. These results suggest that while EGF can stimulate nuclear PKC, it most likely increases PKC of a different type or in a different subnuclear compartment.

PTA activated nuclear PKC to a degree similar to that of ovine prolactin (Table 1). This result is consistent with previous observations showing that tumor-promoting phorbol esters mimic certain aspects of prolactin action in rat liver (3–5, 11) and in Nb2 node lymphoma cells (6, 15, 16, 36). The ability of tumor-promoting phorbol esters to activate PKC correlates positively with their efficacy as promoting agents (15, 37). Moreover, prolactin has been shown to promote the development of preneoplastic lesions in carcinogen-treated rat liver (7). The demonstration now that both PTA and prolactin rapidly activate nuclear PKC suggests that nuclear activation of this kinase represents a general characteristic of tumor-promoting substances.

Insulin, which is regarded as a hepatotrophic hormone (38, 39), did not significantly activate nuclear PKC. Also, no activation was detected in response to a cAMP analogue (Table 1). The synthetic corticosteroid dexamethasone had no detectable effect on nuclear PKC activity.

Antibodies Directed Against Prolactin and the Hepatic Prolactin Receptor Inhibit Prolactin Activation of Nuclear PKC. The effects of anti-ovine or anti-rat prolactin antiserum on prolactin-stimulated activation of nuclear PKC were assessed in the presence of 10^{-10} M homologous prolactin (Table 2). In this experiment, each antiserum abolished the activation of nuclear PKC by the respective prolactin. These data demonstrate the specificity of prolactin for stimulation of nuclear PKC. Also shown in Table 2 is the effect of a monoclonal antibody to the rat hepatic prolactin receptor (40) on the activation of hepatic PKC. The antibody used in this study (E29) inhibited binding of ^{125}I-labeled prolactin by 95% when added at 200 μg/ml to either rat liver microsomes or soluble prolactin receptors (40). Addition of the antibody at 200 μg/ml abrogated the stimulatory effect of prolactin on nuclear PKC (Table 2). These data strongly suggest that activation of nuclear PKC by prolactin is a prolactin receptor-mediated phenomenon at the level of the nucleus. Furthermore, the demonstration of the existence of several enzymes and substrates associated with diacylglycerol production in the rat liver nucleus (41) and the recent report describing the

Table 2. Antibodies to prolactin and to the rat liver prolactin receptor inhibit prolactin-dependent activation of nuclear PKC

Treatment	PKC, pmol phosphate per min per mg protein
Vehicle control	1 ± 0.8
Ovine prolactin	185 ± 47*
+ Antiserum to ovine prolactin	0.3 ± 0.2
+ Antibody to rat prolactin receptor	0.2 ± 0.2
Rat prolactin	61 ± 7*
+ Antiserum to rat prolactin	0.2 ± 0.1

Rat liver nuclei were incubated for 3 min at 37°C in the presence of ovine or rat prolactin (10^{-10} M) with or without the addition of anti-prolactin antiserum (1:750 dilution) or the monoclonal antibody to rat hepatic prolactin receptor (200 μg/ml). PKC activity was determined in triplicate, and data represent the mean ± SEM of three separate experiments.
*$P < 0.01$ vs. vehicle control.

synthesis of inositolphospholipids in nuclei of Friend cells (42) suggest that the biochemical machinery necessary for activation of PKC is present in the nucleus.

Effect of Pharmacological Inhibitors on Prolactin-Stimulated PKC Activation in Rat Liver Nucleus. Inhibitors of PKC were assessed for their effects on the activation of nuclear PKC by prolactin (Table 3). Low micromolar concentrations of either H-7 or sphingosine inhibited the ability of prolactin to activate PKC in the nucleus. Importantly, the addition of cyclosporine, an antagonist of prolactin binding and prolactin-stimulated macromolecular synthesis (4, 43, 44), completely blocked activation of nuclear PKC by prolactin (Table 3). The ability of PKC antagonists to block prolactin activation of this nuclear kinase at concentrations shown to be effective at inhibiting PKC in other systems (45, 46) demonstrates that PKC present in the rat liver nucleus is susceptible to pharmacological manipulation in a similar manner to PKC present in other cellular compartments.

Nuclear Site of Action for Prolactin in Tissues Where It Has Mitogenic Action. The dramatic activation of hepatic nuclear PKC in response to prolactin or PTA suggests that other cell types in which prolactin promotes mitogenic activity might also utilize a nuclear activation mechanism. Prolactin has been implicated in the regulation of mitogenesis in lymphoid tissues. Prolactin administration to rats reverses hypophysectomy-induced immunosuppression (47–49). Human and rodent lymphocytes have specific high-affinity prolactin receptors that are blocked by cyclosporine (43, 44, 50–52). Recently it has been reported by Russell and coworkers (50, 53–56) that prolactin is required for mitogenesis in murine splenocytes. Further, murine splenocytes make and secrete a 46-kDa lymphocyte prolactin in response to concanavalin A stimulation, and mitogenesis can be blocked totally by the addition of anti-prolactin antiserum (55, 56). An explosion of other reports corroborates the finding that prolactin modulates immunity in a variety of immune responses (44, 57–62). Therefore, it was important to test whether prolactin would activate PKC in nuclear preparations of splenic mononuclear cells. The addition of 10^{-10} M prolactin to rat splenocyte nuclei resulted in a dramatic elevation of PKC activity within 3 min (Table 4). Kuo and coworkers (28) report an exceedingly high level of PKC in spleen, even higher than in brain tissue. The ability of prolactin to activate PKC in apparently pure nuclear fractions of both liver and splenic mononuclear cells, tissues for which prolactin is a known dose-dependent mitogen, strongly suggests that PKC-mediated mitogenesis is regulated at the nuclear level by appropriate hormones and growth factors. Further, the synergistic ability of prolactin and EGF, both mitogenic agents for liver, to activate nuclear PKC provides an interaction mechanism for liver mitogenesis similar to the platelet-derived growth factor/EGF/insulin-like growth factor I effects demonstrated for mitogenesis of fibroblasts (63).

Table 3. Pharmacological inhibitors of prolactin binding and PKC activation abolish lactogen-stimulated nuclear PKC activity

Treatment	Inhibitor conc., μM	PKC, pmol phosphate per min per mg protein
Vehicle control	—	1.0 ± 0.8
Ovine prolactin	—	225 ± 14*
+ H-7	6	28 ± 28
+ Sphingosine	20	0.6 ± 0.5
+ Cyclosporine	1	0.8 ± 0.6

Rat liver nuclei were incubated for 3 min at 37°C in the presence of 10^{-10} M prolactin with or without the addition of the antagonists indicated. PKC activity was determined in triplicate, and data are expressed as the mean ± SEM of three separate experiments.
*$P < 0.01$ vs. vehicle control.

Table 4. Prolactin activates nuclear PKC in tissues where it has mitogenic action

Tissue	PKC*
Rat liver nuclei	1.0 ± 0.6
+ Ovine prolactin	230 ± 19†
Rat splenocyte nuclei	5 ± 2
+ Ovine prolactin	717 ± 58†

Nuclei were incubated in the presence or absence of prolactin (10^{-10} M) for 3 min at 37°C. PKC activity was determined in triplicate, and the data are expressed as the mean ± SEM of three separate experiments.
*PKC activity is expressed as pmol of phosphate per min per mg of protein for liver nuclei and as pmol of phosphate per min per 4 × 10^5 nuclei for splenocyte nuclei.
†$P < 0.01$ vs. respective vehicle control.

The implications of this paper are important for our understanding of nuclear function of polypeptide hormones such as prolactin. Previously, we reported that prolactin, as well as partial hepatectomy, which has been linked to an increase in circulating prolactin, caused an apparent translocation of PKC to the particulate fraction in rat liver (13). In view of the present report, increased PKC activity in this hepatic fraction may reflect prolactin-mediated activation of nuclear PKC, since nuclear components are present in the 106,000 × *g* pellet in which PKC activity was assessed. The ability of PTA to mimic the effects of prolactin on nuclear PKC activation suggests that stimulation of nuclear PKC may be a general mechanism by which tumor-promoting substances mediate a pleiotropic response. In our study, only polypeptide hormones that stimulate liver mitogenesis activated nuclear PKC. This suggests that activation of nuclear PKC by internalized hormones or growth factors represents an initial signalling mechanism for phosphorylation of key nuclear proteins, resulting in new RNA and protein synthesis. Of further significance, the immunosuppressive cyclic peptide cyclosporine completely inhibited prolactin activation of nuclear PKC. We have hypothesized that the immunosuppressive properties of cyclosporine are related to its ability to antagonize prolactin action in lymphocytes, a system in which prolactin serves as a comitogen (53–56). The present report provides evidence that this agent may exert its antiproliferative effects at the level of the cell nucleus, where it interferes with prolactin receptor interactions. A nuclear site of action for prolactin opens the door for numerous future studies of phospholipid metabolism, phosphorylation patterns, and new gene expression.

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