THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 274, No. 52, Issue of December 24, pp. 37499–37505, 1999

Printed in U.S.A.

Activation of PKC δ in the Rat Corpus Luteum during Pregnancy

POTENTIAL ROLE OF PROLACTIN SIGNALING*

(Received for publication, July 13, 1999)

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Maintenance of pregnancy in the rat requires the corpus luteum. At a time when rat placental lactogens (rPLs) are required to support progesterone production by the corpus luteum and when relaxin expression is initiated, expression of a specific protein kinase C (PKC) isoform, PKC δ, is dramatically increased. We therefore assessed whether prolactin (PRL) receptor activation promotes activation of PKC δ in a luteinized granulosa cell model. We also assessed the activation status of PKC δ in corpora lutea obtained when the corpus luteum is exposed to chronically high concentrations of rPLs. The activity of PKC δ was assessed by two means: an immune complex (IC) assay and Western blotting with a phospho-epitope-specific antibody that detects PKC δ phosphorylated on serine 662. PKC δ activation in the IC kinase assay was determined by the ability of immunoprecipitated PKC δ to phosphorylate the PKC δ-preferential substrate small heat shock protein (HSP-27). Treatment of luteinized rat granulosa cells with phorbol myristate acetate, a known activator of PKC, promoted a 7-fold increase in HSP-27 phosphorylation by PKC δ. Similarly, immunoreactivity with the phospho-epitope-specific PKC δ antibody was increased in extracts prepared from luteinized granulosa cells treated with phorbol myristate acetate or following *in vitro* activation of recombinant PKC δ. Using these assays, we assessed whether PRL receptor agonists were capable of activating PKC δ in luteinized granulosa cells. PRL receptor agonists induced translocation PKC δ from the cytosolic to the Triton-soluble membrane fraction and increased PKC δ activity assessed by both IC kinase assay and Western blotting with phospho-epitope-specific PKC δ antibody. Analysis of PKC δ activity in corpora lutea obtained during pregnancy by both the IC kinase assay and Western blotting with the phospho-epitope-specific PKC δ antibody revealed that PKC δ activity was increased throughout the second half of pregnancy. These results demonstrate that PRL receptor activation promotes the acute activation of PKC δ in luteinized rat granulosa cells. At a time when the rat is exposed to chronically high concentrations of rPLs, PKC δ is increasingly expressed and active.

The corpus luteum is a transient endocrine gland of the ovary formed following ovulation by the differentiation of granulosa and thecal cells (1). In the rat, the corpus luteum is the sole source of the progesterone that is necessary to maintain pregnancy to term and is thus necessary throughout pregnancy (1). It is therefore of great interest to assess the signal transduction pathways employed within the corpus luteum that are involved in the regulation of its function.

PKC[1] is a family of serine/threonine kinases that has been implicated in the regulation of numerous signaling pathways (2, 3). The PKC family consists of 10 different isoforms that have been grouped into three categories based on the structural and functional differences among family members (4). Conventional isoforms α, βI, βII, and γ isoforms are activated by PS, DAG, and Ca^{2+}. Novel isoforms do not require Ca^{2+} for kinase activity and are represented by the δ, ϵ, η, and θ isoforms. The atypical isoforms ζ and ι require only PS for activation.

As the number of PKC isoforms has increased, so has the expectation that distinct PKC isoforms will have distinct functions within a cell. This has been, to some extent, borne out by the specific roles of PKC isoforms in mitogenesis (5, 6), gene expression (5, 7, 8), and secretion (9–12). The ability of a distinct PKC isoform to regulate discrete biological functions is likely due to three factors: (*a*) the requirements for activation of a PKC isoform as determined by that isoform's structure (4); (*b*) the localization of different PKC isoforms to distinct subcellular locales, thus limiting access of a particular PKC isoform to relevant substrates (2); and (*c*) the substrate specificity of PKC isoforms (13–17).

The ovary of the rat has been found to express the same subset of PKC isoforms throughout all the stages of development that have been analyzed (18). These are the conventional isoforms α, βI, and βII, the novel isoforms δ and ϵ, and the atypical isoform ζ. The δ isoform can be distinguished from the other isoforms by the striking increase of both PKC δ mRNA and protein levels in corpora lutea in the second half of pregnancy (19). The rat corpus luteum is maintained in the second half of pregnancy by the combined actions of intraluteal E_2 and PRL-like hormones such as the placenta-derived rPL-1 (1, 20). We have found that rPL-1 treatment of luteinized granulosa cells induces phosphorylation of Stat 3 on both tyrosine 705 and serine 727 and induction of relaxin mRNA expression,[2] a major product of the rat corpus luteum in the second half of pregnancy (21). Both Stat 3 serine phosphorylation and induction of relaxin expression by rPL-1 were abrogated by the PKC δ inhibitor rottlerin.[2]

Based on the ability of the PKC δ inhibitor rottlerin to block

* This work was supported by National Institutes of Health Grant P01 HD 21921 (to M. H.-D.) and the Training Program in Reproductive Biology (T32 HD 07068). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: PKC, protein kinase C; PS, phosphatidylserine; DAG, diacylglycerol; PRL, prolactin; PMA, phorbol myristate acetate; E_2, estrogen; rPL, rat placental lactogen; DMEM/F-12, Dulbecco's modified Eagle's medium/Ham's F-12; T.S., Triton-soluble; IC, immune complex; HSP-27, 27-kDa heat shock protein; $PI(3,4,5)P_3$, phosphatidylinositol 3,4,5-trisphosphate; PI3-kinase, 1-phosphatidylinositol 3-kinase; Stat, signal transducer and activator of transcription.

[2] C. A. Peters, E. T. Maizels, M. C. Robertson, R. P. Shiu, M. S. Soloff, and M. Hunzicker-Dunn, submitted for publication.

rPL-1-induced Stat 3 serine phosphorylation and relaxin mRNA expression, we now seek direct evidence (*a*) that PRL receptor activation by rPL-1 activates PKC δ in a luteinized granulosa cell model and (*b*) that PKC δ is active in an *in vivo* setting in the corpus luteum of pregnancy, coincident with high rPLs in serum of rats. Our results show that signaling through the PRL receptor promotes acute activation of PKC δ in rat luteinized granulosa cells and that the PKC δ in corpora lutea obtained when rPLs are elevated is active. These results thus implicate the PRL signaling pathway in the activation of PKC δ in corpora lutea of pregnancy.

EXPERIMENTAL PROCEDURES

Materials—The following materials were purchased: [γ-^{32}P]ATP (specific activity 3000 Ci/mmol) from NEN Life Science Products; SDS-polyacrylamide gel electrophoresis reagents from Bio-Rad; protein standards from Diversified Biotech (Boston, MA); recombinant HSP-27 from Stressgen Biotechnology (Victoria, British Columbia, Canada); Hybond C-extra nitrocellulose and ECL reagents from Amersham Pharmacia Biotech; GF109203X from Alexis (San Diego, CA); purified recombinant PKC δ from Pan Vera (Madison, WI); PKC δ-specific monoclonal antibody directed to the N terminus of PKC δ from Transduction Labs (lot 2, released April 1995) (Lexington, KY). M-4 (PKC α) monoclonal antibody was obtained from K. Leach (The Upjohn Company), and PKC δ serine 662 phospho-epitope-specific antibody was a gift from New England Biolabs (Beverly, MA). All other PKC-specific antisera were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); 4G-10 (anti-phosphotyrosine) monoclonal antibody was purchased from Upstate Biotechnology (Lake Placid, NY). All other biochemical reagents were purchased from Sigma. Final concentrations are indicated throughout.

Granulosa Cell Culture—Rats were obtained at 21 days of age from Charles River Laboratories (Portage, MI) and were maintained in accordance with "Guidelines for the Care and Use of Experimental Animals" by protocols approved by the Northwestern University Animal Care and Use Committee. Follicles were collected from 30-day-old rats that had been administered a low dose of human chorionic gonadotrophin (0.15 IU) given subcutaneously twice daily for 2 days. On the following day, a high dose of human chorionic gonadotrophin (10 IU) was given to rats via tail vein injection, and ovaries were isolated 7 h later (22, 23). Cells were harvested by mechanical dispersion and put into culture by modifications of the method of Bley *et al.* (24) as described by Carr *et al.* (25). The medium used for all procedures was DMEM/F-12 without phenol red and with 15 mM HEPES, 3.15 g/liter glucose, 1% charcoal-stripped fetal bovine serum, 100 IU penicillin G, and 100 μg/ml streptomycin. Following sequential incubations at 37 °C in 6 mM EGTA in DMEM/F-12 and 0.5 M sucrose in DMEM/F-12, ovaries were returned to DMEM/F-12. Granulosa cells were released into the medium from all follicles using 30-gauge needles and gentle pressure. Cells were pelleted at 100 × *g* for 15 min, counted using trypan blue, and plated at a density of approximately 1 × 10^6 cells/ml on plastic dishes. Cells were cultured in humidified atmosphere at 37 °C, 5% CO_2 with 10 nM estradiol-17β (in ethanol, final concentration 0.5%). The medium was changed every 3 days.

Pregnant Rats—Pregnant rats were obtained from Charles River Laboratories. On the appropriate day of pregnancy rats were sacrificed, ovaries were dissected, corpora lutea were removed, and protein was collected from the corpora lutea as specified within.

Protein Preparation and Western Immunoblot Analysis—Subcellular fractions of cell or tissue extracts were prepared by homogenization in protease/phosphatase inhibitor-enriched homogenization buffer (10 mM potassium phosphate, pH 7.0, 1 mM EDTA, 5 mM EGTA, 10 mM $MgCl_2$, 2 mM dithiothreitol, 1 mM sodium vanadate, 80 mM β-glycerophosphate, 100 μg/ml pepstatin A, 10 μg/ml leupeptin, 40 μg/ml phenylmethylsulfonyl fluoride) followed by centrifugation at 105,000 × *g* for 70 min. The soluble fraction was removed and the pellet resuspended in the same buffer adjusted to 0.1% Triton X-100 and incubated with stirring for 60 min followed by centrifugation at 105,000 × *g* for 30 min. Alternatively, clarified cell lysates were prepared by homogenization in a lysis buffer (10 mM potassium phosphate, pH 7.0, 1 mM EDTA, 5 mM EGTA, 10 mM $MgCl_2$, 2 mM dithiothreitol, 1 mM sodium vanadate, 50 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 0.5% Nonidet P-40, 0.1% deoxycholic acid) followed by centrifugation at 20,000 × *g* for 20 min. Samples were denatured by adding 3× stop (3% SDS, 150 mM Tris-HCl, 2.4 mM EDTA, 3% β-mercaptoethanol, 30% glycerol, and 0.5% bromphenol blue) followed by heating for 5 min at 100 °C. Protein concentrations in both fractions were determined (26) using bovine serum albumin as a standard. Protein samples were separated by SDS-polyacrylamide gel electrophoresis and transferred to membranes for Western blot analysis. Western blot analysis was performed using the ECL detection system (Amersham Pharmacia Biotech) following the protocol provided by the manufacturer. Where appropriate, membranes were stripped of antibodies according to the protocol provided with the ECL detection system. Densitometric quantitation was performed by image analysis using a Bio-Rad Molecular Analyst or BioImage Intelligent Quantifier software.

IC Kinase Assay—Cells were cultured for 9 days with E_2 and subsequently treated with 10 nM PMA or ethanol vehicle for 10 min or with 5 μg/ml rPL-1 for 5 min. Clarified cell lysates or subcellular fractions of cell extracts were prepared, and PKC δ or control immunoprecipitations were performed on samples containing 500 μg of total protein. Antibody-antigen complexes were precipitated by further incubation with an anti-mouse Ig antibody, where applicable, and protein A-conjugated Sepharose or with protein A/G-conjugated agarose alone. Pelleted proteins were washed with low salt (10 mM Tris-HCl, pH 7.2, 150 mM NaCl, 1% deoxycholate, 1% Triton X-100, 0.1% SDS, 1 mM sodium vanadate, and 40 μg/ml phenylmethylsulfonyl fluoride) and high salt (10 mM Tris-HCl, pH 7.2, 1 mM NaCl, 0.1% Nonidet-P40, 1 mM sodium vanadate, and 40 μg/ml phenylmethylsulfonyl fluoride) radioimmunoprecipitation assay (RIPA) buffer. Immunoprecipitates were resuspended in 50 μl of TE (10 mM Tris, pH 7.5, 0.1 mM EGTA). Precipitated proteins were subjected to an *in vitro* kinase assay in a final volume of 110 μl (containing 45 μM α-glycerophosphate (pH 7.0), 9 mM $MgCl_2$, 0.9 mM dithiothreitol, 4.5 μM ATP, 5 μCi of [γ-^{32}P]ATP, and 5 μg of exogenous substrate). Where indicated, the PKC inhibitor GF109203X (bisindolylmaleimide) was added at a final concentration of 5 μM. Incubations were typically for 5 or 10 min (unless otherwise indicated) at 37 °C, and reactions were terminated by adding 50 μl of 3× stop and heat denaturation. Proteins in the samples were separated by SDS-polyacrylamide gel electrophoresis, and the top half of the gel, containing PKC δ, was transferred to a membrane and subjected to Western blotting while the bottom half of the gel, containing exogenous substrate, was dried and exposed to film to detect incorporation of labeled phosphate. Alternatively, the entire gel was transferred, and phosphorylation was detected by exposure to film followed by Western blotting. A similar procedure was employed to analyze PKC δ activation during pregnancy. Pregnant rats were sacrificed on the indicated day of pregnancy, and ovaries were isolated. Corpora lutea were isolated and homogenized as described above for use in the IC kinase assay. Where indicated, kinase assay included lipids (PS (45 μg/ml) and 1,2-diolein (1.6 μg/ml)).

In Vitro Phosphorylation of PKC δ—Reactions were conducted as described above with 3.5 nM recombinant PKC δ replacing immunoprecipitated PKC δ.

RESULTS

PKC Isoform Activation during Pregnancy—To begin to assess the activation of PKC isoforms during pregnancy, we employed the fact that translocation to a membrane fraction is widely recognized as an index of activation of PKC for many isoforms (2). Subcellular fractions of corpora lutea from days 11, 18, and 21 of pregnancy were prepared and the cytosol and T.S. (membrane) fractions analyzed by Western blot analysis. Results depicted in Fig. 1 show that all PKC isoforms expressed are partially active at some time during the second half of pregnancy based on their presence in the T.S. fraction. PKCs α and ε are both detected in the T.S. fraction on days 11 and 21 of pregnancy and to a reduced extent on day 18 of pregnancy. In contrast, PKC βII is detected in the T.S. fraction predominately on day 18. PKC ζ is detected in the T.S. fraction only on day 21 of pregnancy. PKC δ exhibits the previously described increase in expression (19), and increased amounts of PKC δ are detected in the T.S. fraction as pregnancy progresses to term.

PKC δ IC Kinase Assay—Because luteal PKC δ exhibits an increase in expression during pregnancy (19) and appears to be partly activated throughout the second half of pregnancy, we sought to analyze PKC δ activity more closely. To this end we employed an assay that involves the immunoprecipitation of PKC δ. The kinase activity of the precipitated PKC δ is then assessed by its ability to phosphorylate *in vitro* HSP-27, a PKC δ preferential substrate (17). This assay is conducted in the

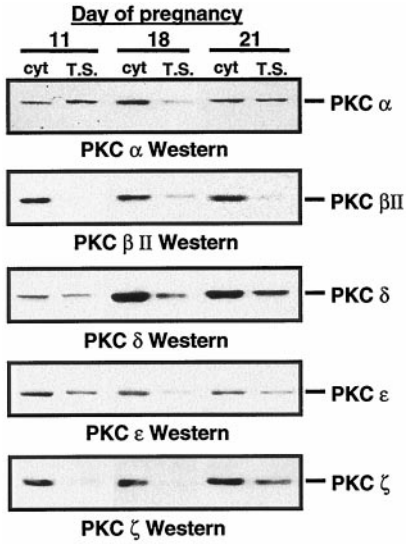

FIG. 1. **Localization of PKC isoforms to the Triton-soluble fraction in corpora lutea of pregnant rats.** Rat corpora lutea were isolated on the indicated day of pregnancy, homogenized, and subcellular fractions (*cyt*, cytosol; *T.S.*, Triton-soluble) collected. PKC isoform Western blots were performed as indicated to assess translocation of the isoforms to the T.S. subcellular fraction indicative of activation. The same blot was stripped and reprobed to assess the translocation of each PKC isoform. The results are representative of three separate experiments.

absence of exogenous activators so that the kinase activity that is measured reflects that which was attained in the cell or tissue.

Fig. 2*A* shows the results of an IC kinase assay performed on samples from luteinized granulosa cells. Cells were treated with either vehicle or 10 nM PMA for 10 min. The results of PKC δ immune precipitations from cytosol and T.S. fractions reveal that PMA promotes both the translocation of PKC δ from the cytosol to the T.S. fraction and the partial down-regulation of PKC δ (Fig. 2*A, top panel*; compare amount of PKC δ in *lane 1* with that in *lanes 3* and *4*). PMA also induced the tyrosine phosphorylation of the PKC δ translocated into the T.S. fraction (Fig. 2*A, second panel*). Tyrosine phosphorylation of PKC δ has been observed by several groups to be a consequence of PKC δ activation, especially in response to PMA, but the function of PKC δ tyrosine phosphorylation is not yet fully understood (5). The autophosphorylation of PKC δ on serine/threonine residues during the *in vitro* kinase assay (Fig. 2*A, third panel*) mirrors the amount of PKC δ immunoprecipitated in each lane. Phosphorylation of the exogenous substrate HSP-27 by immunoprecipitated PKC δ from vehicle and PMA-treated cells is shown in the *bottom panel* of Fig. 2*A*. Although PKC δ exhibits activity in the cytosolic fraction of control cells (*lane 1*), phosphorylation of HSP-27 by PKC δ is clearly enhanced in the T.S. fraction of PMA-treated cells consistent with PKC δ translocation to this fraction (*lane 4*). PMA-stimulated activation of PKC δ is most clearly appreciated when the amount of phosphorylated HSP-27 is assessed relative to the amount of PKC δ that is immunoprecipitated (Fig. 2*B*). Results of this analysis show that PMA-dependent PKC δ activation is readily detected by this IC kinase assay.

We further evaluated the characteristics of the *in vitro* PKC δ IC kinase assay. Cells were treated with 10 nM PMA for 10 min and then homogenized in a membrane extracting buffer. PKC δ was immunoprecipitated, and the IC kinase assay reaction was performed for 1–10 min. The *upper panel* of Fig. 3*A* is a PKC δ Western blot that shows that equivalent amounts of PKC δ were immunoprecipitated. The *lower panel* shows that

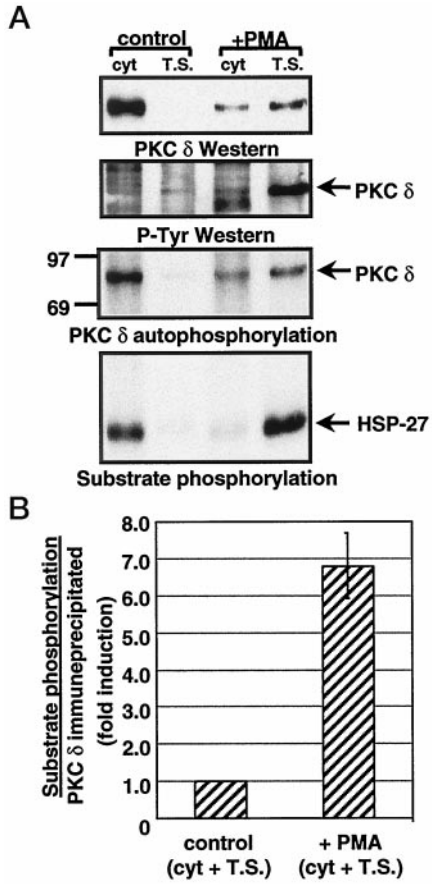

FIG. 2. **PKC δ IC kinase assay following activation with PMA.** *A*, luteinized granulosa cells were cultured in the presence of E_2 for 9 days and subsequently stimulated with 10 nM PMA or vehicle for 10 min; subcellular fractions were prepared and IC kinase assay performed as described under "Experimental Procedures." Both PKC δ autophosphorylation (*third panel*) and phosphorylation of exogenous substrate (*bottom panel*) were detected by autoradiography. PKC δ immunoprecipitation (*top panel*) and tyrosine phosphorylation (*second panel*) were detected by Western blotting. The migration of molecular mass markers (in kDa) is indicated at the *left*. The position of HSP-27 is indicated on the *right* of the *bottom panel,* and the positions of tyrosine-phosphorylated and autophosphorylated PKC δ are indicated on the *right* of the *second* and *third panels,* respectively. *B*, average fold induction (±S.E.) of HSP-27 phosphorylated per unit of PKC δ immunoprecipitated by PMA from seven experiments (autoradiographic detection of phosphorylated HSP-27/density of PKC δ antibody immunoreactivity from Western blots) over vehicle-treated cells.

HSP-27 phosphorylation increases with time of incubation. When PKC δ antibody is omitted, PKC δ is not immunoprecipitated and HSP-27 is not phosphorylated (Fig. 3*B*). HSP-27 phosphorylation by immunoprecipitated PKC δ is nearly undetectable when the *in vitro* reaction is performed in the presence of the PKC inhibitor GF109203X (27, 28) (Fig. 3*C*). Taken together, these results show that the PKC δ IC kinase assay detects authentic activation of PKC δ attained in PMA-treated luteinized granulosa cells.

PKC δ IC Kinase Assay during Pregnancy in Corpora Lutea of Rats—Based on our evidence that the IC kinase assay readily detects active PKC δ and utilizing this assay, we sought to analyze the activity of PKC δ in corpora lutea obtained during the second half of pregnancy. PKC δ was immunoprecipitated from corpora lutea collected from days 11, 18, and 21 of pregnancy and homogenized in a membrane extracting buffer. The amount of PKC δ immunoprecipitated from these days of pregnancy (Fig. 4*A, top panel*) correlates with the increase in PKC δ expression previously observed (19). Tyrosine phosphorylation of PKC δ is also observed, particularly on

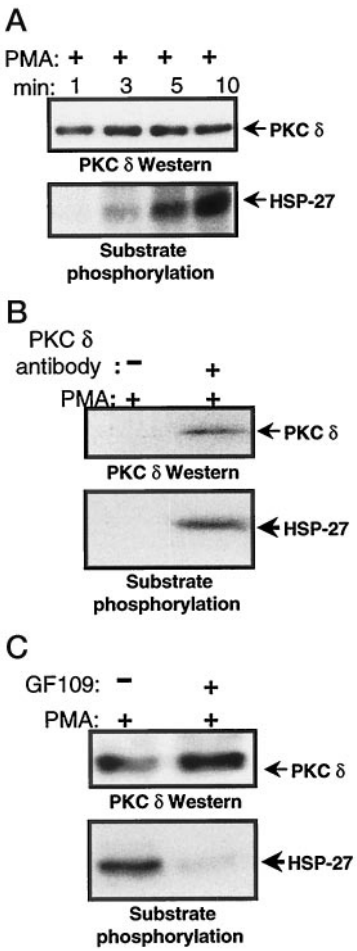

FIG. 3. **Phosphorylation of HSP-27 in IC kinase assay increases with time of *in vitro* incubation, is not detected in the absence of PKC δ immunoprecipitation, and is blocked by *in vitro* treatment with a PKC inhibitor.** *A*, cells were treated with PMA and prepared for IC kinase assay as described for Fig. 2, except that membrane extracts were prepared and the length of *in vitro* incubation following PKC δ immunoprecipitation was varied from 1 to 10 min as indicated. The results are representative of three experiments. *B*, cell treatment and IC kinase assay are as described for *A*, except that PKC δ antibody was either present (+) or not present (−) in the immunoprecipitation as indicated. The results are representative of three experiments. *C*, cell treatment and IC kinase assay are as in *A*, except PKC inhibitor GF109203X was either present (+) or absent (−) in the *in vitro* reaction following the immunoprecipitation of PKC δ as indicated. The position of HSP-27 is indicated. The results are representative of four experiments.

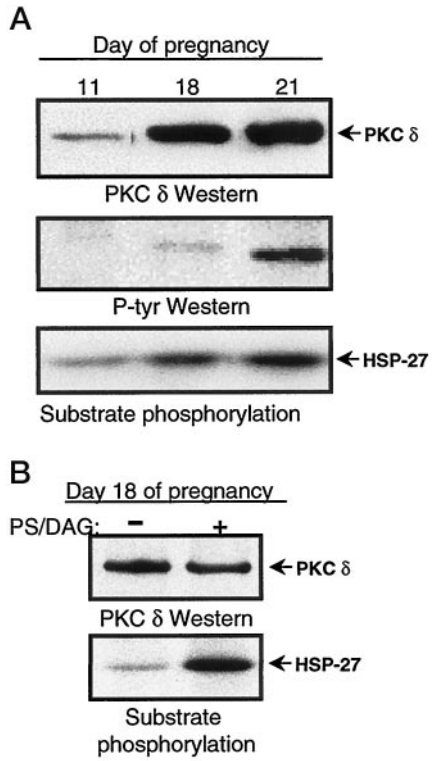

FIG. 4. **PKC δ IC kinase assay during pregnancy confirms luteal PKC δ is active in the second half of pregnancy, but activity is further stimulated by the addition of PKC activators *in vitro*.** *A*, proteins from rat corpora lutea obtained on the indicated days of pregnancy were collected in a membrane-extracting buffer, and IC kinase assay was performed. Phosphorylation of exogenous substrate (*bottom panel*) was detected by autoradiography. PKC δ immunoprecipitation (*top panel*) and tyrosine phosphorylation (*middle panel*) were detected by Western blotting. The results are representative of five experiments. *B*, IC kinase assay from day 18 of pregnancy was performed essentially as described under "Experimental Procedures"; however, *in vitro* reaction was performed in either the absence (−) or presence (+) of the PKC activators PS and DAG, as indicated. Phosphorylation of exogenous substrate (*lower panel*) was detected by autoradiography. PKC δ immunoprecipitation (*upper panel*) was detected by Western blotting. The results are representative of three experiments.

day 21 of pregnancy. Consistent with the translocation analysis shown in Fig. 1, HSP-27 phosphorylation in the IC kinase assay is detected in each of the luteal samples and increases as pregnancy progresses (Fig. 4*A, bottom panel*). These results suggest that PKC δ is indeed active throughout the second half of pregnancy, as predicted from the results presented in Fig. 1. PKC δ exhibits similar activity in corpora lutea obtained on days 18 and 21. To determine whether this level of PKC δ activity reflects maximal activation of PKC δ, we assessed the activity of PKC δ in an IC kinase assay upon addition of the PKC activators PS and DAG. Results show that PKC δ immunoprecipitated from corpora lutea on day 18 of pregnancy can be further activated *in vitro* when PS and DAG are added to the *in vitro* reaction (Fig. 4*B*).

Activation of PKC δ Leads to Phosphorylation of Serine 662—Autophosphorylation of PKC δ on serine 643 is reported to be important for the regulation of PKC δ activity (29). However, mutation of this serine to an alanine did not abolish PKC δ autophosphorylation or activity (29, 30). Serine 662 of PKC δ has also been hypothesized as a site of autophosphorylation because of corresponding autophosphorylation sites on PKC α (serine 657) and PKC βII (serine 660). Using an epitope-specific antibody that reacts with PKC δ phosphorylated on serine 662, we sought to assess whether serine 662 autophosphorylation occurs coincident with activation of PKC δ. The time-dependent activation *in vitro* of recombinant PKC δ by PS and DAG is shown (Fig. 5*A*). PKC δ exhibits increased histone phosphorylation and autophosphorlyation with time of incubation, as shown in the *lower two panels* of Fig. 5*A*, and a corresponding increase in immunoreactivity as detected with the PKC δ serine 662 phospho-epitope-specific antibody (Fig. 5*A, top panel*). A PKC δ Western blot confirms that equivalent amounts of PKC δ are present in each lane (Fig. 5*A, top panel*).

We also evaluated the ability of the PKC δ serine 662 phospho-epitope-specific antibody to detect PMA-dependent PKC δ activation in luteinized granulosa cells. Luteinized granulosa cells were stimulated with 10 nM PMA or vehicle for 10 min. Results show that the phosphorylation of PKC δ on serine 662 is also increased following PMA-dependent activation of PKC δ in luteinized granulosa cells (Fig. 5*B*). PKC δ exhibits some basal activity, based on phospho-epitope-specific antibody immunoreactivity in the absence of PMA treatment, consistent with the results shown in Fig. 2.

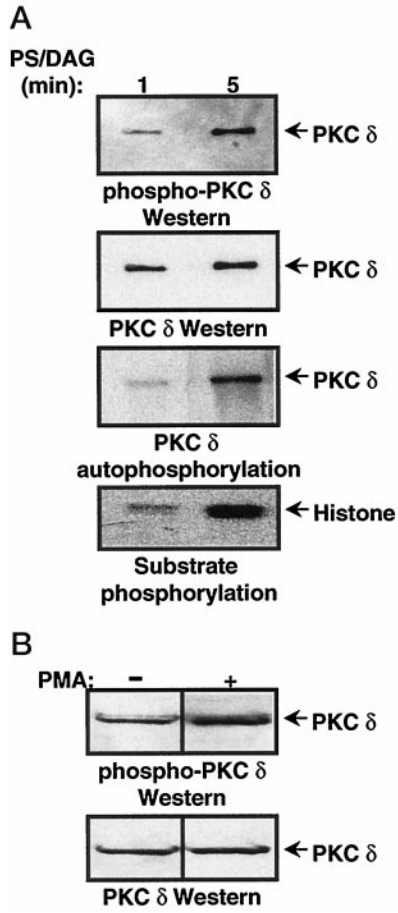

FIG. 5. **Detection of PKC δ activation assessed by increased immunoreactivity with an antibody that detects PKC δ phosphorylated on serine 662 following treatment with PS and DAG *in vitro* or PMA *in vivo*.** *A*, recombinant PKC δ was employed in an *in vitro* kinase assay as described under "Experimental Procedures" for 1–5 min. PKC δ autophosphorylation (*third panel*) or phosphorylation of histone H1 (*bottom panel*) were detected by autoradiography, and Western blotting was performed to detect PKC δ phosphorylated on serine 662 (*top panel*) followed by detection of total PKC δ (*second panel*). The results are representative of three experiments. B, luteinized granulosa cells were cultured in the presence of E_2 for 9 days and subsequently stimulated with 10 nM PMA (+) or vehicle (−) for 10 min; extracts were prepared in a membrane-extracting buffer. Following SDS-polyacrylamide gel electrophoresis, proteins were transferred to nitrocellulose membranes for Western blotting, performed to detect PKC δ phosphorylated on serine 662 (*top panel*), followed by detection of total PKC δ (*second panel*). The results are representative of two experiments.

PKC δ Autophosphorylation on Serine 662 Increases in Corpora Lutea as Pregnancy Progresses—To further confirm the activation of PKC δ in rat corpora lutea during pregnancy, Western blotting with the serine 662 phospho-epitope-specific antibody was performed on extracts prepared from corpora lutea obtained on day 11, 18, or 21 of pregnancy. The increase in PKC δ expression is again apparent (Fig. 6*, lower panel*). Immunoreactivity with the serine 662 phospho-epitope-specific antibody is equivalent on day 18 and 21 of pregnancy, and both are clearly increased compared with the reactivity seen on day 11 of pregnancy (Fig. 6*, upper panel*). Thus, the relative activity of PKC δ detected in the corpora lutea of pregnancy by both membrane translocation (Fig. 1) and IC kinase assay (Fig. 4) is mirrored by reactivity with the serine 662 phospho-epitope-specific antibody.

Activation of PKC Isoforms by PRL—During the second half of pregnancy, when we detect activated PKC δ, the rat corpus luteum is maintained exclusively by the combined actions of intraluteal E_2, aromatized from androgens provided by the placenta (1), and PRL-like hormones such as the placenta-derived rPL-1 (1, 20). We have found that rPL-1 treatment of luteinized granulosa cells promotes phosphorylation of Stat 3 on tyrosine 705 and serine 727 and induction of relaxin mRNA expression,[2] a major product of the rat corpus luteum in the second half of pregnancy (21). Both of these effects of rPL-1 were blocked by the PKC δ inhibitor rottlerin.[2] Based on these results, we considered that PRL receptor activation was a likely candidate to activate PKC δ and possibly other PKCs. We therefore assessed whether or not PKC δ or other PKC isoforms are activated by PRL receptor agonists rPL-1 and PRL.

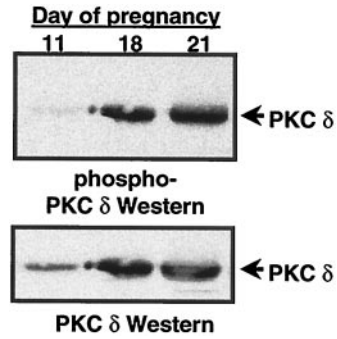

FIG. 6. **PKC δ is increasingly phosphorylated on serine 662 as pregnancy progresses.** Extracts from rat corpora lutea obtained on the indicated days of pregnancy were prepared in a membrane-extracting buffer. Western blotting was performed to detect PKC δ phosphorylated on serine 662 (*upper panel*) followed by detection of total PKC δ (*lower panel*). The results are representative of three experiments.

To this end, luteinized granulosa cells were treated with PRL for 1 or 10 min, and subcellular fractions were prepared. The ability of PRL to induce translocation of the PKC isoforms to the T.S. fraction was assessed by Western blot analysis. Results show that PRL induces the translocation of all PKC isoforms to the T.S. fraction (Fig. 7); however, the extent and time-course of translocation exhibits striking isoform-selective differences. PKC α, βII, and δ translocate to the T.S. fraction 1 min after PRL treatment. Translocation of PKC ε is slower, whereas PKCs βI and ζ exhibit minimal translocation to the T.S. fraction.

PKC δ IC Kinase Assay and Autophosphorylation on Serine 662 following Treatment of Luteinized Granulosa Cells with rPL-1 or PRL—In the following experiments we assessed the activation of PKC δ by PRL receptor agonists in luteinized granulosa cells by PKC δ IC kinase assay and autophosphorylation of PKC δ on serine 662. Luteinized granulosa cells were treated for 5 min with rPL-1, subcellular fractions were prepared, and PKC δ was immunoprecipitated. Translocation of PKC δ in response to PRL receptor activation is again seen following PKC δ immunoprecipitation (Fig. 8*A, upper panel*). However, rPL-1 activation of PKC δ evidenced by HSP-27 phosphorylation is observed in *both* the cytosol and T.S. fractions (Fig. 8*A, lower panel*). This result points out an advantage of using the IC kinase assay over the typical translocation assay and is not the first report of PKC δ activation independent of translocation (31). Activation of PKC δ relative to PKC δ protein is presented in Fig. 8*B*. These results indicate that PRL or rPL-1 activates PKC δ more than 2-fold when the amount of HSP-27 phosphorylation is adjusted to the amount of PKC δ immunoprecipitated.

Acute activation of PKC δ in luteinized granulosa cells in response to PRL receptor activation was also detected with the serine 662 phospho-epitope-specific antibody (Fig. 9, *upper panel*). The *lower panel* confirms that equivalent amounts of PKC δ are present in both extracts. Similar to the results presented in Fig. 5*B*, PKC δ in these luteinized granulosa cells

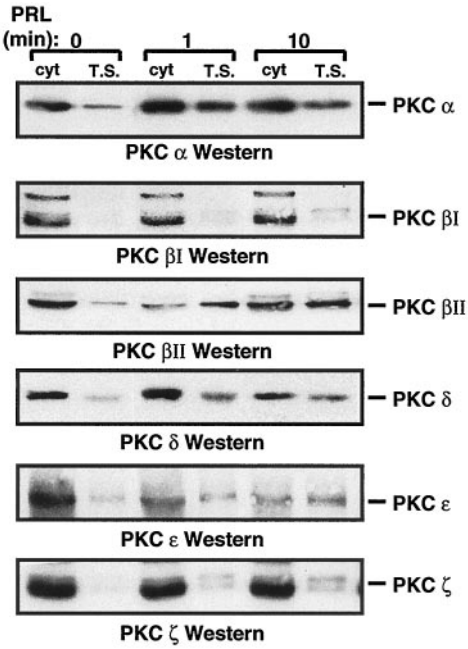

FIG. 7. **Activation of PKC isoforms by PRL.** Luteinized granulosa cells were cultured for 9 days in the presence of E_2 and treated with 5 μg/ml rPL-1 or PRL for the indicated times (*min*), and subcellular fractions were collected. PKC isoform Western blots were performed as indicated to assess translocation of the isoforms to the T.S. subcellular fraction indicative of activation. The same blot was stripped and reprobed to assess the translocation of each PKC isoform. The results are representative of three separate experiments.

exhibits a basal activity based on phospho-epitope-specific antibody immunoreactivity in the absence of PRL treatment.

DISCUSSION

Expression of PKC δ by the rat corpus luteum is dramatically increased coincident with dependence of this structure on chronically elevated levels of rPLs (1, 19). We have also shown that PRL receptor activation promotes relaxin expression by luteinized rat granulosa cells and that PRL-dependent relaxin expression is abrogated by the PKC δ-specific inhibitor rottlerin.[2] This result is consistent with the hypothesis that PRL receptor activation promotes activation of PKC δ. Previous data suggested that PRL is capable of activating PKC in liver, Nb2 lymphoma cells, astrocytes, and vascular smooth muscle cells, based on the partial translocation of PKC to the particulate cell fraction (32, 33) or on the ability of PKC inhibitors to block a PRL-dependent response (34, 35). However, these reports provided no evidence of which PKC isoforms were activated by PRL.

To test the hypothesis that PRL receptor activation leads to activation of PKC δ, we evaluated the activity of PKC δ by three criteria: its translocation from the cytosolic to T.S. membrane fraction, an IC kinase assay of cytosolic and translocated PKC δ, and immunoreactivity with a PKC δ phospho-epitope-specific antibody. Specificity of the IC kinase assay for PKC δ was augmented by use of a PKC δ-preferential substrate, HSP-27. We established that immunoreactivity with the serine 662 phospho-epitope-specific antibody is increased when PKC δ is activated. Serine 662 is a predicted autophosphorylation site on PKC δ (29), and our *in vitro* results using recombinant PKC δ clearly show that serine 662 is an autophosphorylation site. Although the function of autophosphorylation of serine 662 on PKC δ remains to be determined, we have demonstrated that autophosphorylation of this site is a clear marker of PKC δ activation. This conclusion is based on the *in vitro* results using recombinant PKC δ, in which activation by PS and DAG led to

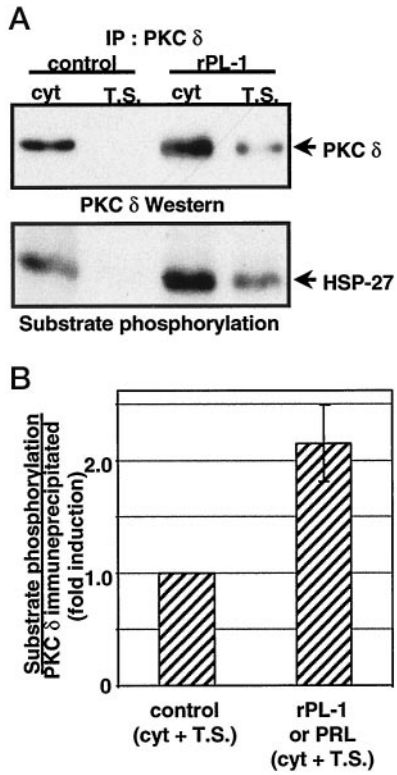

FIG. 8. **IC kinase assay following activation by rPL-1 shows that PKC δ is activated in both the cytosol and triton-soluble fractions.** *A*, cells were treated and prepared for IC kinase assay as described for Fig. 2, except that cells were treated with 5 μg/ml rPL-1 (rPL-1) or vehicle (control) for 5 min. Phosphorylation of exogenous HSP-27 (*lower panel*) was detected by autoradiography. PKC δ immunoprecipitation (*upper panel*) was detected by Western blotting. *B*, average fold induction (±S.E.) of substrate phosphorylated per PKC δ immunoprecipitated by rPL-1 or PRL from five experiments (autoradiographic detection of phosphorylated HSP-27/density of PKC δ antibody immunoreactivity from Western blots) over vehicle-treated cells.

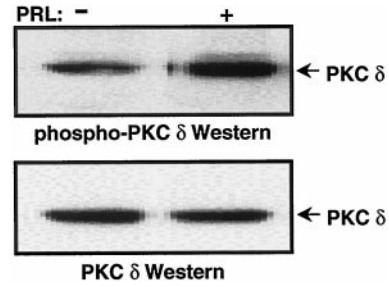

FIG. 9. **Phosphorylation of PKC δ on serine 662 is increased following treatment of luteinized granulosa cells with PRL.** Cells were treated as described for Fig. 5*B*, except that cells were treated with 5 μg/ml PRL (+) or vehicle (−) for 5 min. Western blotting was performed to detect PKC δ phosphorylated on serine 662 (*upper panel*) followed by detection of total PKC δ (*lower panel*). The results are representative of three experiments.

increased histone phosphorylation and serine 662 phosphorylation, as well as on results from luteinized granulosa cells showing increased phosphorylation of serine 662 following PMA-dependent PKC activation.

Utilizing these assays of PKC δ activation, we have shown that PKC δ is activated in corpora lutea exposed to chronically elevated levels of rPLs. By IC kinase and immunoreactivity with the PKC δ serine 662 phospho-epitope-antibody, PKC δ is activated throughout the second half of pregnancy, based on our evaluation of its activity on days 11, 18, and 21 of pregnancy. Despite the fact that PKC δ is increasingly expressed in corpora lutea as pregnancy progresses, PKC δ also appears to

be increasingly activated as pregnancy progresses, based on detection of increased PKC δ in the T.S. fraction. Our results also suggest that translocation analysis may not allow a full appreciation of the PKC activity because IC kinase assay analysis showed that PKC δ activity was increased in both the cytosol and T.S. fractions in response to PRL or rPL-1.

Because the pathway leading to PKC δ activation in the intact corpus luteum of the rat is difficult to assess, we determined whether PKC δ was activated in a luteinized granulosa cell model. Our results demonstrate that signaling through the PRL receptor in response to either PRL or rPL-1 promotes activation of PKC δ. PRL receptor activation induced the translocation of PKC δ from the cytosolic to the T.S. fraction, increased IC kinase activity of PKC δ in both the cytosol and T.S. fractions, and increased immunoreactivity with the serine 662 phospho-epitope-specific antibody. This report represents the first identification of a specific PKC isoform activated by PRL. However, the cellular pathway from the PRL receptor to PKC δ remains to be elucidated. In some cell models PRL causes an increase in intracellular Ca^{2+} (36), consistent with activation of PLC, whereas in rat granulosa cells PRL causes an increase in cellular DAG (32) in the absence of an increase in IP_3/Ca^{2+} (37) consistent with activation of phospholipase D (38). Activation of PKC by PRL might also involve activation by phosphoinositide-dependent kinase 1 via PRL/PI3-kinase (39). PRL can increase the level of $PI(3,4,5)P_3$ in a PI3-kinase-dependent fashion (40). $PI(3,4,5)P_3$ has been shown to activate novel PKC isoforms as well as PKC ζ both *in vitro* and following activation of PI3-kinase (41–43). $PI(3,4,5)P_3$ is also required for the activation of phosphoinositide-dependent kinase 1, which may play a role in activation of PKC by phosphorylating PKC isoforms on their activation loop (44).

PRL receptor activation also induced the translocation of PKCs α and βII and, to a lesser extent, PKCs βI, ε, and ζ to the T.S. fraction. We also detected each of these PKC isoforms in the T.S. fraction of corpora lutea at distinct times during the second half of pregnancy. This result suggests that, as in luteinized granulosa cells, PRL receptor activation not only activates PKC δ but may also activate additional PKCs, such as PKCs α and βII. Additional studies are needed to confirm that translocation of these PKCs to the T.S. fraction reflects their activation.

A number of kinases and transcription factors have been associated with signaling through the PRL receptor, including the Src family kinases Src, Fyn, and Yes, Janus kinase-2, Stats 1,3, and 5, as well as PI3-kinase (45). We have found that Src, Fyn, Yes, Janus kinase-2, Stat 3, and PI3-kinase all co-precipitate with luteal PKC δ during pregnancy.[3] Therefore, it is possible that the PRL receptor serves as a site not only for activation of these signaling pathways and PKC δ but also for the integration of these various signals to induce the appropriate responses within the corpus luteum.

In conclusion, this report shows that PRL receptor activation promotes activation of PKC δ. Because PKC δ in the rat corpus luteum is increasingly expressed and activated as pregnancy progresses at a time when the corpus luteum is exposed to and dependent upon rPLs, our results implicate PKC δ, and perhaps other PKC isoforms as well, in the PRL receptor signaling pathway.

Acknowledgments—The authors thank Josh Cottom for technical support.

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[3] C. A. Peters and M. Hunzicker-Dunn, unpublished observation.

Activation of PKC δ in the Rat Corpus Luteum during Pregnancy: POTENTIAL ROLE OF PROLACTIN SIGNALING

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J. Biol. Chem. 1999, 274:37499-37505.
doi: 10.1074/jbc.274.52.37499