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 phospha-epitope-specific antibody that detects PKC δ phosphorylated on serine 662. PKC δ activity 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 phospha-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 phospha-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 phospha-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.

PKC1 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 Ca2+ novel isoforms do not require Ca2+ for kinase activity and are represented by the δ, ε, η, and θ isoforms. The atypical isoforms ξ and ζ 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 E2 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

1 The abbreviations used are: PKC, protein kinase C; PS, phosphati-dylerine; DAG, diacylglycerol; PRL, prolactin; PMA, phorbol myristate acetate; E2, 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) P3, phosphatidylinositol 3,4,5-trisphosphate; PI-3-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.
soluble fraction was removed and the pellet resuspended in the same DMEM/F-12 without phenol red and with 15 mM HEPES, 3.15 g/liter later (22, 23). Cells were harvested by mechanical dispersion and put was given to rats via tail vein injection, and ovaries were isolated 7 h phin (0.15 IU) given subcutaneously twice daily for 2 days. On the mals" by protocols approved by the Northwestern University Animal cordance with "Guidelines for the Care and Use of Experimental Ani-

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Materials—The following materials were purchased: [γ-32P]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 Biotechnologie (Victoria, British Columbia, Canada); Hybond-C extra nitrocellulose and ECL reagents from Amersham Pharmacia Biotech; GF109203X from Alexis (San Diego, CA); purified re-

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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.

Granulosa Cell Culture—Rats were obtained at 21 days of age from Charles River Laboratories (Portage, MI) and were maintained in ac-

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

Fig. 2A 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. 2A, 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. 2A, 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. 2A, 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. 2A. 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. 2B). 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. 3A is a PKC δ Western blot that shows that equivalent amounts of PKC δ were immunoprecipitated. The lower panel shows that 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. 3B). 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. 3C). 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. 4A, top panel) correlates with the increase in PKC δ expression previously observed (19). Tyrosine phosphorylation of PKC δ is also observed, particularly on...
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. 4A, 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. 4B).

**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. 5A). PKC δ exhibits increased histone phosphorylation and autophosphorylation with time of incubation, as shown in the lower two panels of Fig. 5A, and a corresponding increase in immunoreactivity as detected with the PKC δ serine 662 phospo-epitope-specific antibody (Fig. 5A, top panel). A PKC δ Western blot confirms that equivalent amounts of PKC δ are present in each lane (Fig. 5A, 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. 5B). 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.
**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-equivalent 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-equivalent 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 E₂, aromatized from androgens provided by the placenta (1), and PRL-like hormones such as the placenta-derived rPRL-1 (1, 20). We have found that rPRL-1 treatment of luteinized granulosa cells promotes phosphorylation of Stat3 on tyrosine 705 and serine 727 and induction of relaxin mRNA expression, a major product of the rat corpus luteum in the second half of pregnancy (21). Both of these effects of rPRL-1 were blocked by the PKC δ inhibitor rottlerin. 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 rPRL-1 and PRL.

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 β 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 rPRL-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. 8A, upper panel). However, rPRL-1 activation of PKC δ evidenced by HSP-27 phosphorylation is observed in both the cytosol and T.S. fractions (Fig. 8A, 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. 8F. These results indicate that PRL or rPRL-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. 5B, PKC δ in these luteinized granulosa cells
cells were cultured for 9 days in the presence of E2 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 re-probed to assess the translocation of each PKC isoform. The results are representative of three separate experiments.

expresses 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. 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 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 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 Ca²⁺ (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 IP₃/Ca²⁺ (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)P₃ in a PI3-kinase-dependent fashion (40). PI(3,4,5)P₃ has been shown to activate novel PKC isoforms as well as PKC δ both in vitro and following activation of PI3-kinase (41–43). PI(3,4,5)P₃ 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-purify with luteal PKC δ during pregnancy. 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.

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