Original Paper

Journal of Biomedical Science

J Biomed Sci 1996;3:126–132

M.C. Audy
P. Vacher
A.M. Vacher
B. Dufy

Laboratoire de Neurophysiologie CNRS URA 1200, Université de Bordeaux II, France

Dual Effect of Prolactin on Protein Kinase C Activity in CHO Cells Expressing Functional Prolactin Receptors

Key Words
Chinese hamster ovary cells
Phorbol myristate acetate
Prolactin
Protein kinase C
Tyrosine kinase

Abstract
We investigated the effects of prolactin (PRL) on the protein kinase C (PKC activity in Chinese hamster ovary (CHO-E32) cells stably transfected with rabbit mammary gland PRL receptor cDNA. These cells express a functional long form of PRL-R. A 10-min to 2-hour treatment with 5 n*M* PRL resulted in the translocation of PKC activity from the cytosol to the membrane. Longer treatment (10–24 h) with the same concentration of PRL decreased the PKC activity in both particulate and cytoplasmic fractions. The PRL effect was dose dependent: maximal action was obtained with 1–10 n*M*. The PRL-induced activation of PKC was blocked by 20 n*M* staurosporine, a PKC inhibitor. Two inhibitors of tyrosine kinase, herbimycin A (1.75 μ*M*) and genistein (100 μ*M*), had no effect on PRL-induced activation of PKC.

Although the best-known effects of prolactin (PRL) are the somatogenic and lactogenic functions in the establishment of lactation, PRL has been found to regulate a great number of biochemical processes in many other tissues and cells [17, 25]. Although the PRL action mechanism in all these tissues is not known, its first step occurs through the binding of the hormone with specific receptors [18]. The PRL receptor (PRL-R) belongs to the growth hormone receptor family. Two forms of PRL-R have been cloned and sequenced [4, 33], distinguished by the size of their cytoplasmic domain. A physiologically active long form and a short form, the physiological role of which is not yet known, have been described.

The transmembrane signaling mechanism triggered by PRL binding to its receptor is poorly understood. Most of the published works were conducted on Nb2 lymphoma cells that possess a mutant long form of PRL-R [1, 15]. The PRL action in these cells has been related to protein phosphorylation [20], protein kinase C (PKC) activation [8, 38], tyrosine kinase activation [30], cyclic adenosine monophosphate levels and G proteins [21, 36, 37], the Na^+/H^+ exchange system [35, 38], calcium mobilization [8], and phospholipid metabolism [19]. Chinese hamster ovary (CHO) cells provide a good opportunity for studying the action mechanism of PRL. Usually these cells have no PRL-R, but they are easily transfectable. A well-established cell line of CHO cells (CHO-K1) was stably transfected (CHO-E32 cells) with the long form of rabbit mammary gland PRL-R cDNA [24]. Since transfection results in the preservation of functional PRL-R, this cell line is ideal for assessing the PRL transduction mechanism [23]. Elsewhere, we have recently shown that PRL-R binding triggers a rise in intracellular calcium concentration $[Ca^{2+}]_i$ in the CHO-E32 cell line, by stimulating both Ca^{2+} entry and mobilization from intracellular Ca^{2+} stores [40]. PRL-stimulated Ca^{2+} entry was due to the

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1021–7770/96/0032–0126$10.00/0

M.C. Audy, MD
Laboratoire de Neurophysiologie, CNRS URA 1200
Université de Bordeaux II
146, rue Léo-Saignat, BP 22
F–33076 Bordeaux Cedex (France)

Received:
July 10, 1995
Accepted:
November 1, 1995

activation of a Ca^{2+}-dependent K^+ conductance resulting in hyperpolarization of the membrane potential that activates hyperpolarization-driven Ca^{2+} channels [28]. In many systems using Ca^{2+} as second messenger, its mobilization is associated with hydrolysis of polyphosphoinositides leading to the production of inositol 1,4,5-trisphosphate and diacylglycerol which stimulates a phospholipid-calcium-dependent kinase, PKC.

PRL has been shown to activate translocation of PKC from cytosol to particulate in various cell types such as rat aortic muscle cells [32], rat hepatocytes [6], or rat splenocytes [31]. All these results support the hypothesis that PRL action is linked with the generation of second messengers coupled to PKC activation.

In the present study, the PRL action upon intracellular PKC activation in CHO-E32 cells expressing functional PRL-R was investigated. We show that PKC translocation occurs after PRL stimulation of this cell line. Moreover, like other PKC activators, PRL has a dual action: transient stimulation followed by depletion under long-term treatment. However, despite extensive efforts over the past few years, the signal transduction pathway associated with PRL-R activation remains to be elucidated. Recent works from several laboratories have clearly shown that tyrosine kinase activation may be an early event associated with PRL-R activation [20]. The present study does not support the contention that tyrosine kinase activation is a prerequisite step for the activation of PKC by PRL in CHO-E32 cells.

Materials and Methods

Cell Culture

CHO-K1 cells were transfected with PRL-R cDNA (CHO-E32) as previously described [33]. They were maintained in Ham's F12 medium (Seromed, Strasbourg, France) containing 10% fetal calf serum (Gibco, Grand Island, N.Y., USA) at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The medium was changed every 2 or 3 days for 8 days. PRL 5 n*M* (oPRL-19; NIDDK) was incubated with the cells for various lengths of time (10 and 20 min and 2 h, 10, and 18 h). Phorbol myristate acetate (PMA, Sigma Chemical, St. Louis, Mo., USA) was applied to the cells for 18 h (1 μ*M*) in order to downregulate the PKC activity or for 10 min (10 n*M*) as a PKC activator. Staurosporine (20 n*M*; Calbiochem), a PKC inhibitor, was applied for 2 h and then PRL for 2 h. The tyrosine kinase inhibitors genistein (100 μ*M*; Sigma) and herbimycin A (1.75 μ*M*; Gibco) were added for 10 h, and then PRL was applied for 2 h.

Assay of PKC Activity

PKC was assayed using the method reported by Parant and Vial [27]. Briefly, PKC was determined by measuring the incorporation of ^{32}P into histone H_1 type III-S (Sigma) from [γ-^{32}P]ATP (Amersham, France). The cells were scraped and washed before homogenization by soft sonication at 4°C in a buffer consisting of 20 m*M* Tris-HCl, 2 m*M* EDTA, 10 m*M* EGTA, 300 m*M* sucrose, 2 m*M* dithiothreitol, 2 m*M* phenylmethylsulfonylfluoride, 25 μg/ml leupeptin, and 50 μl/ml trans-epoxysuccinyl-*l*-leucylamido(4-guanido)-butane E64 (pH 7.5). After centrifugation at 100,000 *g* for 1 h, the supernatants containing cytosolic fractions were isolated. The cell pellets, resuspended in the above-mentioned buffer containing Triton X-100 (1%) for 30 min and centrifuged [16], constitute the particulate fraction. Samples were collected with a 100-m*M* NaCl elution during DEAE-cellulose purification. The PKC was measured in a total volume of 100 μl containing 20 m*M* Tris-HCl at pH 7.5, 10 m*M* Mg acetate, 50 μ*M* [γ-^{32}P]ATP, 500 μg/ml histone H_1 type III-S, 1 m*M* $CaCl_2$, 1 m*M* dithiothreitol, 80 μg/ml phosphatidylserine, and 8 μg/ml diolein (Sigma). The reaction was stopped after 8 min at room temperature by adding 100 μl of a 40% trichloroacetic acid solution and a mixture of 20 μl 50 m*M* adenosine triphosphate and 5 mg/ml bovine serum albumin. The precipitates were collected on filter glass fiber disks, then washed, and dried automatically using a Multimash 2000 cell harvester. The enzyme activity was defined by subtracting the amount of ^{32}P incorporated into histone in a reaction mixture containing Ca^{2+} and phosphatidylserine from that obtained using a reaction mixture containing 0.5 m*M* EGTA and no Ca^{2+} and no phosphatidylserine. Data were expressed as picomoles ^{32}P incorporated per minute per milligram protein. The protein concentration was measured applying the method of Bradford [5].

Statistical Analysis

The results are expressed as mean values ± SEM or as a percentage of particulate PKC/total PKC (particulate + cytosolic) ± SEM. Statistical comparisons were performed by one-way analysis of variance and Fisher's PLSD as posttests. Student's t test was used when appropriate. $p < 0.01$ was considered significant.

Results

Evaluation of PKC Distribution

Table 1 summarizes data concerning PKC activity in CHO-E32 cells cultured with or without PRL or PMA. We used 5 n*M* PRL because we had already shown that this concentration was effective on $[Ca^{2+}]_i$ and ion channel activity in CHO-E32 cells. In control cells, about 20% of PKC activity was localized in the particulate fraction and 80% in the cytosolic fraction. PMA (10 n*M*, 10 min), a potent PKC activator, induced a significant increase (about 300%) in PKC translocated to particulate. PRL (5 n*M*, 10 min) also induced an (about 200%) increase in particulate PKC activity (table 1). When PMA was used for PKC depletion (1 μ*M*, 18 h), PKC was undetectable in both cytosolic and particulate fractions. A long-term treatment with PRL (5 n*M*, 18 h) showed an identical result. When PKC was downregulated with PMA, PRL (5 n*M*, 10 min) was unable to provoke PKC activation.

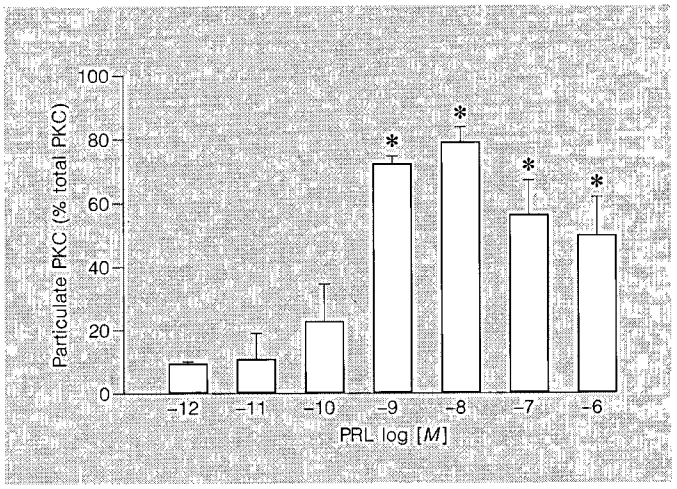

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Fig. 1. Dose-response effect of PRL on the PKC activity. CHO cells were incubated with increasing PRL concentrations as indicated. The values shown are the mean ± SEM of four or five determinations using histone as exogenous substrate, as indicated in Material and Methods. *Significantly different from controls ($p < 0.01$).

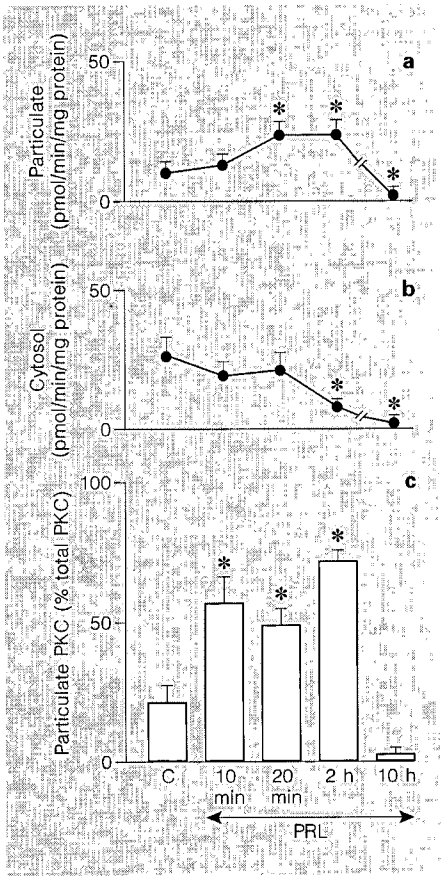

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Fig. 2. PKC distribution according to the duration of PRL treatment. PKC activity in particulate (**a**) and cytosolic (**b**) fractions before (C, n = 15) and after 5-n*M* PRL treatment for 10 min (n = 7), 20 min (n = 13), 2 h (n = 14), and 10 h (n = 4) expressed as picomoles per minute per milligram protein. Data from the same experiments are represented as the percentage of particulate PKC activity in comparison with total PKC activity (**c**).

Table 1. Effects of long-term (18 h) and short-term (10 min) treatments with 10 n*M* or 1 μ*M* PMA and/or 5 n*M* PRL on PKC distribution in CHO-E32 cells (mean ± SEM).

	Particulate PKC (% total PKC)
Controls	21.18 ± 6.13 (n = 15)
PMA 10 n*M* 10 min	78.86 ± 7.14[a] (n = 7)
PRL 5 n*M* 10 min	60.10 ± 10.4[a] (n = 7)
PMA 1 μ*M* 18 h	<0.01 (n = 7)
PRL 5 n*M* 18 h	<0.01 (n = 5)
PMA 1 μ*M* 18 h + PRL	<0.01 (n = 5)

[a] Significantly different from controls ($p < 0.01$).

Dose Dependency of PRL-Stimulated PKC Activity

Cytosolic and particulate PKC activities were assessed by preincubation of CHO cells with PRL at increasing concentrations: the percentage particulate PKC of total (particulate plus cytosolic) PKC is represented in figure 1. A detectable particulate PKC activity in particulate and in cytosolic fractions occurred in response to 1 and 10 p*M* and 0.1 n*M* PRL as in control cells. An important significant increase in particulate PKC was detected with 1 and 10 n*M* PRL (72.3 ± 3.7 and 78.1 ± 10.9%, respectively, versus 22.7 ± 5.6% for controls; $p < 0.01$). Higher concentrations (100 n*M* and 1 μ*M* PRL) induced a slightly significant ($p < 0.01$) increased PKC activation.

Time Course of the PRL Effect on PKC Activation

A 5-n*M* PRL treatment was applied to CHO cells for 10 and 20 min and for 2 and 10 h (fig. 2).

Between 10 and 20 min of PRL treatment, no significant effect on cytosolic PKC activity appeared (19.1 ± 4.3 and 20.8 ± 5.5 vs. 25.6 ± 5.7 p*M*/min/mg protein; $p > 0.01$). At 2 h the PKC translocation became maximal.

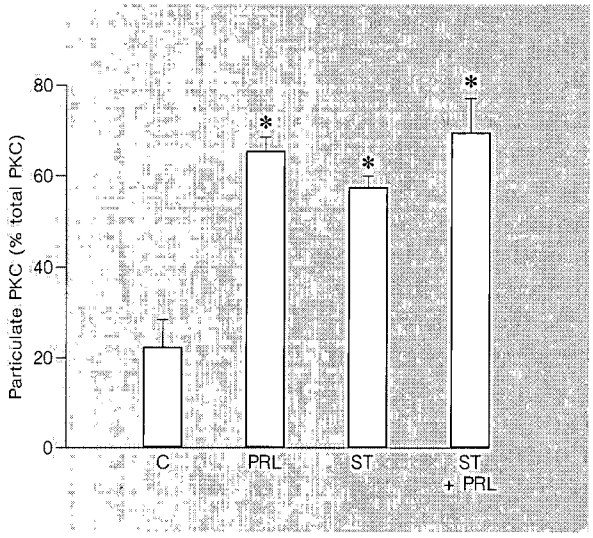

Fig. 3. Effects of staurosporine, a PKC inhibitor, on basal and PRL-stimulated PKC activity. A treatment with 20 n*M* staurosporine (ST, n = 7) for 4 h did not significantly modify the basal PKC activity (C, n = 9). Conversely, the same pretreatment with staurosporine (ST + PRL, n = 7) prior to the 5-n*M* PRL stimulation (2 h, n = 14) completely abolished the PRL action (PRL). *Significantly different from controls ($p < 0.01$).

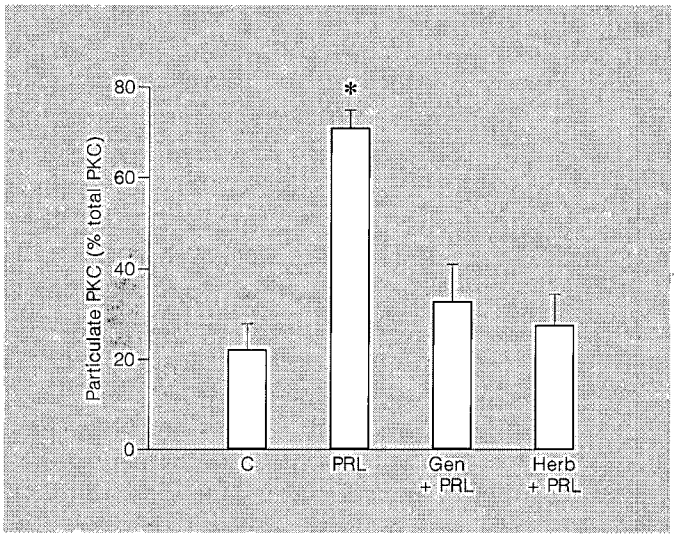

Fig. 4. Effects of two inhibitors of tyrosine kinase, herbimycin A and genistein, on PRL-induced PKC activity. A pretreatment with 1.75 μ*M* herbimycin A (Herb, n = 7) or 100 μ*M* genistein (Gen, n = 8) for 10 h prior to stimulation with 5 n*M* PRL for 2 h did not significantly modify the PRL response. *PRL versus control (C) $p < 0.01$.

and the cytosolic PKC was significantly reduced (8.4 ± 2.1 vs. 25.6 ± 5.7 p*M*/min/mg protein; $p < 0.01$). After 10 h, the cytosolic activity was very low (1.1 ± 0.1 p*M*/min/mg protein).

The particulate-associated PKC was significantly higher between 20 min (19.5 ± 7.8 vs. 11.1 ± 4.6 p*M*/min/mg protein; $p < 0.01$) and 2 h (22.7 ± 5.6 vs. 11.1 ± 4.6 p*M*/min/mg protein; $p < 0.01$) after PRL treatment. A 10 h PRL treatment showed the initial step of downregulation previously observed after an 18-hour treatment (0.8 ± 0.02 vs. 25.6 ± 5.7 p*M*/min/mg protein; $p < 0.01$).

These long-term PRL treatments caused a decrease in PKC activity in both particulate and cytosolic fractions.

PKC Inhibitor Action on PRL-Induced PKC

To clarify the complex action of PRL on PKC distribution, a potent PKC inhibitor, staurosporine, was tested. Staurosporine (20 n*M*) was applied for 4 h with or without PRL (5 n*M*, 2 h; fig. 3). Staurosporine alone showed no effect on particulate-associated PKC (31.9 ± 8.1 vs. 21.2 ± 6.2% for controls). When PRL was added for 2 h, staurosporine blocked the activation of PKC which had previously been observed, and particulate PKC did not increase (27.5 ± 7.1% for PRL plus staurosporine vs. 70.6 ± 3.9% for PRL alone; $p < 0.01$).

Tyrosine Kinase Inhibitor Action on PRL-Induced PKC

The signal transduction pathway of PRL involves tyrosine kinase activation [30]. To test a possible downstream activation of PKC by tyrosine kinase, the potent tyrosine kinase inhibitors genistein and herbimycin A were used (fig. 4). Neither genistein (100 μ*M*) nor herbimycin A (1.75 μ*M*), applied to CHO-E32 cells 10 h before PRL treatment (5 n*M*, 2 h), caused any difference in PKC responses: 63.2 ± 4.8% for PRL, 64.1 ± 2.3 for genistein ($p > 0.01$), and 77.8 ± 2.2% for herbimycin A ($p > 0.01$). Genistein (100 μ*M*) or herbimycin A (1.75 μ*M*) alone had no effect on PKC activation in CHO cells (data not shown).

Discussion

PRL receptor cDNAs have recently been cloned from tissues of several species: humans [3], rats [4], and rabbits [12]. CHO cells have been cotransfected with rabbit cDNA of the physiologically active long form of PRL-R and a gene containing the promoter of the ovine β-lactoglobulin coding for chloramphenicol acetyltransferase [24]. It has been shown [23] that PRL-R transfected under

these conditions was still capable of being activated by PRL and that this cell line (CHO-E32) is, therefore, a valuable tool for studying the PRL transduction mechanism. Although many studies have implicated the PRL-R function [17, 18], the events following binding of PRL to its receptors are poorly understood. Several potential components of the signal transduction pathway have been implicated. Cyclic adenosine monophosphate has been shown to block PRL-stimulated Nb2 cell mitogenesis [20]. Recently, PRL-stimulated Nb2 cell mitogenesis has been coupled with cholera and pertussis toxin action [21, 36, 37], supporting the contention that G proteins may be implicated in PRL function. PKC has been described as playing a crucial role in the signal transduction pathway [26]; PKC is thought to be the second messenger for PRL action in many tissues [6, 8, 31, 32, 38]. In Nb2 lymphoma cells where the PRL action was also associated with the activation of PKC [29], an accumulation of PKC in the particulate fractions was observed after 4 and 12 h of PRL treatment, with a maximal increase after 6 h. In rat aortic smooth muscle cells, PRL induced a dose-dependent increase in both particulate and cytosolic PKC [32], with an optimal increase at 10 p*M*. In rat liver cells, the effects of PRL have been linked with an increase in 1,2-diacylglycerol and subsequent PKC activation [6]. Administration of PRL produced a rapid (30 min) increase in PKC from rat hepatocyte nuclei, with an optimal increase at 0.1 n*M* for ovine PRL and 1 p*M* for rat PRL [7]. In addition, Villalba et al. [41] have shown that PRL induced a Ca^{2+} mobilization from intracellular stores in these cells. All these studies were performed using cells provided with spontaneous PRL-R. Recently, we have demonstrated that, in CHO-E32 cells, PRL action implicates an increase in $[Ca^{2+}]_i$ originating from both extracellular and intracellular calcium sources [40]. However, to our knowledge, no study has shown an effect of PRL on PKC activity in these cells. In the present study, we showed that:

(1) Intracellular PKC regulation was preserved in CHO cells expressing PRL-R. PMA stimulation provoked a clear increase in particulate PKC activity, while a long-term treatment with high concentrations of PMA downregulated the PKC activity.

(2) When PRL was added to CHO cells expressing PRL-R, a significant increase in percent particulate PKC/total PKC was observed within 10 min; this increase was maximal after 2 h. This kinetics is intermediate between the rapid effect (maximum at 30 min) observed in hepatocytes [7] and the slower effect (maximum at 6 h) found in Nb2 cells [29]. Interestingly, a long-term treatment with the same concentration of PRL (5 n*M*) downregulates the PKC activity as does long-term treatment with PMA. This type of dual effect of PRL on PKC activity was described by Sauro et al. [32] who speculated that high PRL concentrations might downregulate the PKC activity in rat aortic smooth muscle cells, whereas it had been stimulated at lower concentrations. A dose-dependent response to PRL stimulation was also observed with optimal concentrations of PRL of 1 and 10 n*M*. The higher concentrations effective in CHO cells as compared with rat aortic cells [32] or rat hepatocyte nuclei [7] are probably due to a lower number of PRL-R at the CHO cell surface. The specificity of PRL action was supported by the use of a PKC inhibitor, staurosporine, which abolished the PRL-induced PKC activation. The fact that staurosporine had no effect on basal PKC activity suggests that, under our experimental conditions, PKC was not activated in the absence of PRL. All current studies, including our own, support the contention that PKC is involved in PRL action. PKC implication in PRL function in CHO cells is also borne out by observations that PRL cannot activate the PKC activity in cells PKC depeleted by PMA. Alternatively, PMA treatment may destroy the integrity of the PRL receptor.

(3) Under the present experimental conditions, the tyrosine kinase pathways did not seem to interfere with PKC activation in CHO cells expressing PRL-R. PKC is a serine/threonine kinase activated by Ca^{2+}, phospholipids, and diacylglycerol. Thus, PKC is generally thought to be activated by signal transduction events producing diacylglycerol. The effect of PRL on PKC activation in mouse mammary tissue [13] has been described as involving a tyrosine kinase activation. We tested the effects of tyrosine kinase inhibitors on PRL-induced PKC activation to determine whether the same phenomenon occurs in CHO cells. Herbimycin A was shown to specifically inactivate tyrosine kinase in vitro at various concentrations: 3 mg/ml for 2 h [14, 39] and 0.1 mg/ml for 18 h [22]. Neither genistein nor herbimycin A (1 mg/ml for 10 h) decreased the PRL-induced PKC in our experiments. This observation indicates that tyrosine kinases do not seem to be implicated in PRL-induced PKC activation in CHO cells, since, under our conditions, neither tyrosine kinase inhibitor reduced the magnitude of PRL-induced PKC responses.

The link between the previously described effects of PRL on $[Ca^{2+}]_i$ and those of PRL on the PKC activity presented here remains to be determined. Serine/threonine protein kinases like PKC are known to be capable of phosphorylating membrane proteins such as the proteins

forming ion channels. However, the effect of these phosphorylations on ion channel activity is somewhat controversial and seems to be dependent on the cell type. In the rat pituitary GH3 cell line, phorbol esters activate voltage-dependent calcium channels [11, 34]. On the other hand, PMA-stimulated PKC activity inhibits the same channels in neurons [9, 10]. In GH3 cells, a 12-*o*-tetradecanoylphorbol-13-acetate-induced increase in $[Ca^{2+}]_i$ has been described as being due to an indirect action on a K^+ channel [2]. We have shown that PRL inhibits an *L*-type voltage-dependent Ca^{2+} channel in CHO-E32 cells, but stimulates the Ca^{2+} influx by activating a Ca^{2+}-dependent, phosphorylable K^+ conductance that hyperpolarizes the membrane potential [28]. The present study shows that PKC may be involved in such a process. The effects of PKC activators and blockers on these conductances are currently under investigation in our laboratory.

It is now generally recognized that hormone stimulation involving phosphatidylinositol hydrolysis by phospholipase C usually associates diacylglycerol and IP3 formation. The signaling role, if any, of the numerous compounds formed during the metabolism of IP3 has yet to be demonstrated in the CHO cell line. Although we have shown that PRL induces Ca^{2+} mobilization from intracellular stores, we have, at present, no data implicating IP3 in this phenomenon. On the other hand, diacylglycerol production and subsequent PKC activation may occur independently of IP3 production, by the action of a phospholipase D on phosphatidylcholine.

In summary, in the present study we have shown that short-term treatment of CHO-E32 cells with PRL stimulates PKC activity independently of tyrosine kinase activation, whereas long-term treatment downregulates the PKC activity, as does PMA. This dual action of PRL on PKC activation may be taken into account in the understanding of the complex mechanism of PRL action.

Acknowledgments

We would like to thank J. Djiane (INRA, Jouy-en-Josas) for providing the CHO cells transfected with PRL-R cDNA (CHO-E32), M.F. Odessa for cell cultures, and D. Varoqueaux for the illustrations. This research was supported in part by grants from Etablissement Public Régional (Aquitaine), Association pour la Recherche sur le Cancer (ARC), and Inserm External Research Contract No. 930704.

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